Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression.

Dissertation submitted as part of fulfilment for the M.D. (Branch-IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R.Medical University, to be held in April-2016

CERTIFICATE

This is to certify that the dissertation entitled, **"Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression"** is the bonafide work of Dr. V. J. Subha toward the M.D **(Branch – IV Microbiology)** Degree examination of the Tamil Nadu Dr. M. G. R. Medical University, to be conducted in **April-2016.**

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I hereby declare that this M.D Dissertation entitled "Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression" is the bonafide work done by me under the guidance of Dr. Rajesh Kannangai, Professor and Head, Department of Clinical Virology, Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

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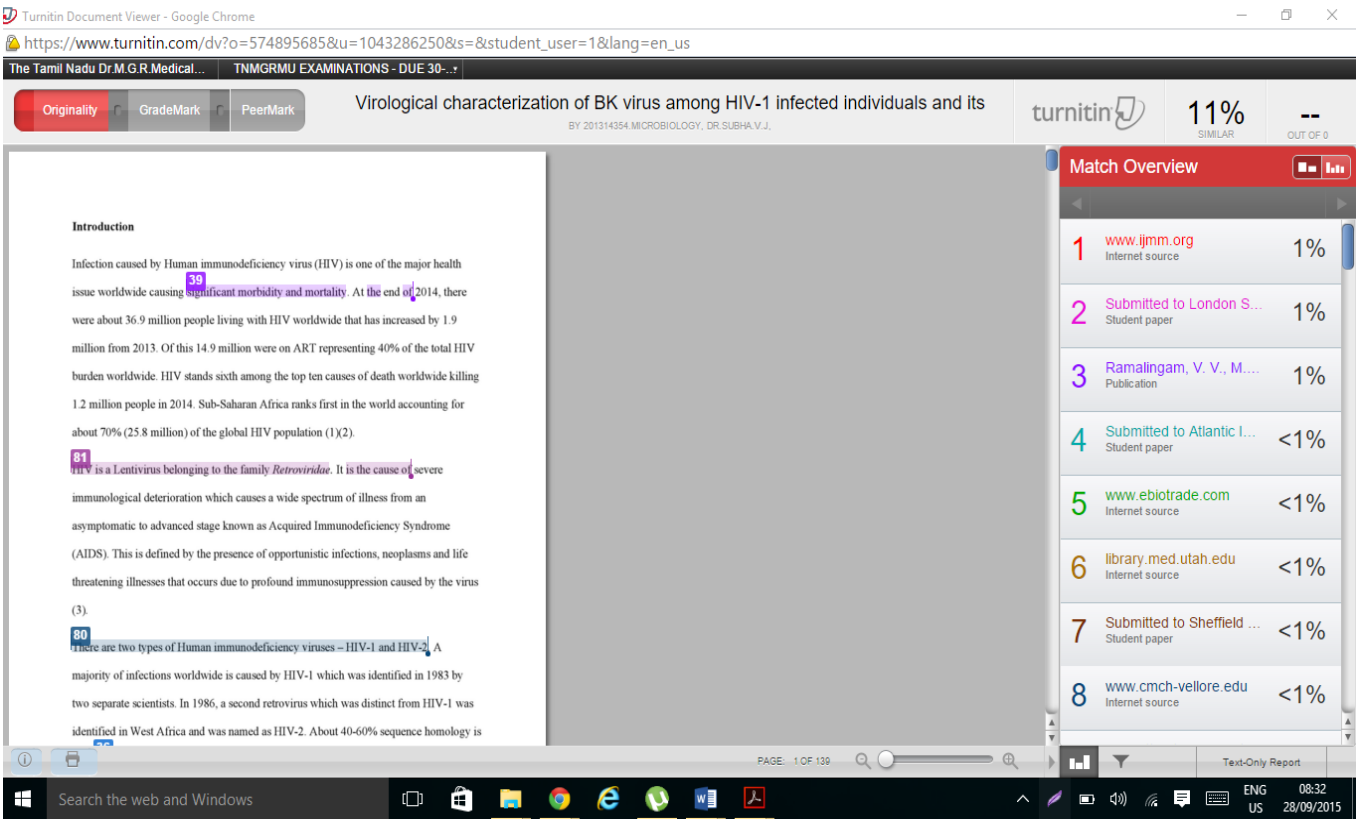
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Introduction
Infection caused by Human immunodeficiency virus (HIV) is one of the major health issue worldwide causing significant morbidity and mortality. At the end of 2014, there were about 36.9 million people living with HIV worldwide that has increased by 1.9 million from 2013. Of this 14.9 million were on ART representing 40% of the total HIV burden worldwide. HIV stands sixth among the top ten causes of death worldwide killing 1.2 million people in 2014. Sub-Saharan Africa ranks first in the world accounting for about 70% (25.8 million) of the global HIV population (1)(2).
HIV is a Lentivirus belonging to the family Retroviridae. It is the cause of severe immunological deterioration which causes a wide spectrum of illness from an asymptomatic to advanced stage known as Acquired Immunodeficiency Syndrome (AIDS). This is defined by the presence of opportunistic infections, neoplasms and life threatening illnesses that occurs due to profound immunosuppression caused by the virus (3).
There are two types of Human immunodeficiency viruses – HIV-1 and HIV-2. A majority of infections worldwide is caused by HIV-1 which was identified in 1983 by two separate scientists. In 1986, a second retrovirus which was distinct from HIV-1 was identified in West Africa and was named as HIV-2. About 40-60% sequence homology is
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Contents

TITLE OF THE STUDY: Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression

DEPARTMENT: Clinical Virology

NAME OF THE CANDIDATE: Dr. V. J. Subha

DEGREE AND SUBJECT: MD, Microbiology

NAME OF THE GUIDE: Dr. Rajesh Kannangai, Professor and Head, Department of Clinical Virology, Christian Medical College, Vellore.

INTRODUCTION:

The human Polyomavirus BK virus (BKV) is an opportunistic pathogen which causes significant morbidity and mortality in immunocompromised population. As HIV causes significant immunosuppression, it is important to determine the frequency and viral load of BK virus which are usually increased in these individuals.

OBJECTIVES:

The main objective was to quantitate BK virus in urine and whole blood of treatment naïve HIV-1 infected individuals and to correlate the viral load with the degree of immunosuppression. The other objectives were to look for any NCCR rearrangements in BKV DNA positive urine and whole blood samples and to determine the genotype of

these samples. The final objective was to prospectively follow up a proportion of BK virus positive individuals and see the effect of antiretroviral treatment on BK viral load by estimating the load following a minimum of 3 months on ART.

METHODOLOGY:

BKV DNA detection was done using In house qualitative TaqMan real time PCR on urine and whole blood samples collected from 187 treatment naïve HIV-1 infected individuals and 93 healthy HIV negative healthy individuals. Samples which were positive by qualitative PCR were subjected to an in house quantitative PCR to determine the BK viral load. All BKV PCR negative samples were subjected to an in-house ERV-3 quantitative PCR to check the DNA integrity. Estimation of CD4 count was done on all blood samples. HIV RNA estimation was done on all BKV positive and a proportion of BKV negative plasma samples. DNA sequencing of the Non-coding Control Region (NCCR) was done on a proportion of BK positive samples to look for any rearrangements. The same samples were genotyped based on NCCR region and 5 randomly selected samples were genotyped based on VP1 region.

RESULTS:

Out of the 187 paired urine and blood samples from HIV-1 infected individuals, 46 (25.6%) urine samples and 2 (1%) blood samples were positive for BK virus. The BKV load range from 1-359886 copies/ml. Only 10 (10.7%) urine samples from healthy controls were positive for BK virus. All BKV PCR negative samples were positive for ERV-3. Out of the 42 urine samples subjected to NCCR sequencing, only 2 samples

showed a rearranged NCCR with the rest of them being archetypal variant. All samples genotyped based on NCCR and VP1 region were genotype I. Out of the 8 follow up individuals on ART, 4 were negative for BK virus, 2 had decreased and 2 had increased BK viral load than the pre ART viral load.

CONCLUSION:

The frequency of BK viruria in HIV-1 infected individuals is higher than healthy controls. There is a strong association of BKV viruria with the extent of immunosuppression. The frequency and viral load of BKV among HIV infected individuals is very low unlike in transplant settings. NCCR rearrangements were not associated with high BK viral load in urine or clinically severe disease.

Keywords: BK virus, HIV, immunosuppression, NCCR, genotype,

1. INTRODUCTION

Infection caused by Human immunodeficiency virus (HIV) is one of the major health issue worldwide causing significant morbidity and mortality. At the end of 2014, there were about 36.9 million people living with HIV worldwide that has increased by 1.9 million from 2013. Of this 14.9 million were on ART representing 40% of the total HIV burden worldwide. HIV stands sixth among the top ten causes of death worldwide killing 1.2 million people in 2014. Sub-Saharan Africa ranks first in the world accounting for about 70% (25.8 million) of the global HIV population (1)(2).

HIV is a Lentivirus belonging to the family *Retroviridae.* It is the cause of severe immunological deterioration which causes a wide spectrum of illness from an asymptomatic to advanced stage known as Acquired Immunodeficiency Syndrome (AIDS). This is defined by the presence of opportunistic infections, neoplasms and life threatening illnesses that occurs due to profound immunosuppression caused by the virus (3).

There are two types of Human immunodeficiency viruses – HIV-1 and HIV-2. A majority of infections worldwide is caused by HIV-1 which was identified in 1983 by two separate scientists. In 1986, a second retrovirus which was distinct from HIV-1 was identified in West Africa and was named as HIV-2. About 40-60% sequence homology is seen between HIV-1 and HIV-2 (4). The rate of transmission and the extent of clinical disease are much lower with HIV-2 than HIV-1 (5).

HIV-1 is transmitted by various modes such as sexual, parenteral by exposure to infected body fluids and mother to child transmission (3). HIV-1 targets the immune cells especially CD4+ T helper cells which acts as the receptor for the virus. After entry, there is a rapid and persistent replication, depleting this subset of T cells by various direct and indirect mechanisms. As there is an imbalance between the T cell production and destruction, intense immunosuppression occurs. This paves way for the emergence of opportunistic pathogens to cause various diseases (6).

Opportunistic infections usually occur in individuals with depleted immune system, mediated by HIV. Irrespective of the CD4 count any individual who is diagnosed to have an opportunistic infection falls into the advanced stage of HIV known as AIDS (7). The common opportunistic infections are

- Bacterial infections mainly pulmonary and extra pulmonary tuberculosis.
- Fungal infections such as Candidiasis, Coccidioidomycosis, Cryptococcosis, Histoplasmosis
- Viral infections like retinitis, oral hairy leukoplakia, EBV associated non-Hodgkin's lymphoma, progressive multifocal leucoencephalopathy, HHV-8 associated Kaposi's sarcoma
- Protozoal infections such as chronic Cryptosporidiosis and Cystisosporiasis

Apart from these defined infections virtually any infectious agent can cause disease in HIV, attributing to the immunosuppression produced by the virus. Thus HIV infected individuals can acquire newer viral co-infections or they can be reactivated from a latent site and cause

severe morbidity and mortality (8). BK virus is one such virus which can get reactivated in immunosuppressed HIV-1 infected individuals..

BK virus is a DNA virus which belongs to the *Polyomaviridae* family. The other important member of this family is the JC (John Cunningham) virus, both of which were discovered in early 1970s. BK virus was isolated from urine of a renal transplant patient, after whom it was named (9). Polyomaviruses are non-enveloped viruses, about 45nm in size with circular double stranded DNA with an icosahedral symmetry containing 72 capsomeres (3) The viral genome has three regions namely the early gene region that codes for the regulatory proteins, the late gene region coding for the capsid proteins and the non-coding control region (NCCR) which harbors the origin of replication and transcription promoters. The latter is prone for rearrangements which is found to be associated with a clinically severe disease and elevated viral load (10).

Four genotypes of BK virus have been described based on the VP1hypervariable region. The defined BKV genotypes are I, II, III, and IV with genotype I being the most common type worldwide and genotype IV reported commonly from East Asia and Europe (11) BKV genotyping can also be done based on NCCR sequences, where four genotypes have been described (I, II, III, and IV) (12).

The initial infection with BK virus occurs during childhood through respiratory route. It may be an asymptomatic infection or can present with a mild respiratory illness. After this the virus remains latent in the renal epithelial cells and various other sites like peripheral blood mononuclear cells (13,14).

The cell mediated immunity plays an important role in limiting the BK viral replication. Thus the virus remains in a quiescent state with intermittent viruria (7%) in immunocompetent individuals (15). It gets reactivated in various immunocompromised states especially in the transplant settings leading to significant nephropathy in renal transplant recipients and hemorrhagic cystitis in hematopoietic stem cell transplant patients (16).

As HIV causes depletion of CD4 cells producing profound immunosuppression, reactivation of BK virus occurs with higher rates of about 20-50% viral shedding is seen in urine of these individuals (14) There are case reports of hemorrhagic cystitis, renal failure and rare reports of meningoencephalitis, retinitis and pneumonia in HIV patients (17–19). Reports from our country have shown 9.3% BKV positivity in renal biopsy specimens with viruria and viremia being 15.7 and 25% after renal transplantation (20,21). The progression of disease correlates with the degree of immunosuppression (8). The major modes of diagnosis for BK virus associated diseases are histopathological examination of renal biopsy tissue for cytopathic changes, detection and quantitation of viral nucleic acid in urine and plasma by molecular methods and cytological examination of urine for decoy cells (22).

The key element in the treatment of BKV associated disease is the reduction of immunosuppression either by withdrawal of the offending drug or decreasing the dosage of the drug (22). With respect to HIV, the most effective treatment would be the control of HIV with Highly Active Antiretroviral therapy (HAART) (18,23)

The main aim of this study was to analyze the frequency of BKV from whole blood and urine of ART naïve HIV infected individuals and correlating the HIV-1 viral load with CD4 count. The study also aims at determining the BK virus genotype and to look for NCCR rearrangements in a proportion of urine samples and all blood samples positive for BK virus. A proportion of BK positive individuals on antiretroviral therapy for a minimum of 3 months were also followed up to determine the effectiveness of ART on clearing the BK virus in these individuals.

1. AIM AND OBJECTIVES

Aim

To quantitate and characterize BK virus in HIV-1 infected individuals and to correlate with the degree of immunosuppression.

Hypothesis

There exists a difference in the BK viral load in urine and blood of HIV -1 infected individuals and this viral load is also associated with the extent of immunosuppression.

Objectives

1. To quantitate BK virus DNA load in blood and urine of treatment naïve HIV-1 infected individuals
2. To correlate BK viral load in urine and whole blood of these individuals with CD4 counts and clinical findings (HIV WHO disease stage I, II, III, IV)
3. To identify the genotype of BK virus in HIV-1 infected individuals
4. To look at the sequence variation in the NCCR (non-coding control region) of BK virus between the strains isolated from blood and urine
5. To prospectively follow up a proportion of BK virus positive individuals and see any effect of antiretroviral treatment on BK viral load by estimating the load following a minimum of 3 months on ART.

3. REVIEW OF LITERATURE

3.1 HIV and AIDS

3.1.1 Discovery

Earliest cases of AIDS (Acquired Immunodeficiency Syndrome) were reported way back in 1970s when the causative agent of the disease was a mystery. The new syndrome was characterized by generalized lymphadenopathy, opportunistic infections and unusual cancers with marked depletion of CD4+ T cells. This was brought to the public notice by Centre for Disease Control (CDC) in 1981 (24) Thereafter two separate groups of scientists under Dr. Luc Montaigner in Pasteur Institute, Paris and Dr. Robert Gallo in National institute of health isolated a virus from patients with AIDS presenting with generalized lymphadenopathy. They named it as HTLV-III and LAV (Lymphadenopathy associated virus) respectively. Simultaneously, a third group under Dr. Jay Levy in the University of California, San Francisco described the same virus in 1984 and called it ARV (AIDS associated Retrovirus). The virus was initially thought to be a variant of Human T Lymph tropic virus (HTLV) which was just then discovered by Dr. Gallo, was later proved to be a different retrovirus belonging to the subfamily of lentiviruses, known as Human Immunodeficiency Virus (HIV) (25).

3.1.2 Epidemiology

3.1.2.1 Global scenario

According to WHO, 36.9 million people were living with HIV worldwide at the end of 2014. This has increased by 2 million from 2013 with Sub-Saharan Africa being the most affected region accounting for about 70% of the global burden of HIV. Deaths due to AIDS related causes was 1.2 million which has decreased from 1.6 million in 2012.This is attributed to the outreach of effective ART and decline in the death rate due to AIDS related causes. In 2014, 40% (14.9 million) of PLHIV were receiving ART of which 13.5 million were from low and middle income countries (1).

3.1.2.2 Indian scenario

India ranks third in the world and largest in Asia with respect to HIV prevalence. Based on the prevalence of HIV in antenatal mothers, it was recorded to be as low as 0.3%, which was actually misleading. Based on the geographical division, 118 districts had prevalence >1% among antenatal mothers with the highest prevalence seen in states Madhya Pradesh, Uttar Pradesh, West Bengal, Orissa, Rajasthan and Bihar (26,27). The four main states which account for about 57% of India's HIV burden are Andhra Pradesh. Karnataka, Maharashtra and Tamilnadu (28)

As of 2013, there were 21 lakh people living with HIV in India accounting for about 37% of deaths due to AIDS related causes worldwide (29). Majority of these people are between 15-40 years of age and women accounting for about 39% of all infections (26). The

prevalence of HIV is about 6-8 times more common in high risk behaviour groups than the general population (30). This group constitutes the female commercial sex workers (2.7%), men having sex with men (4.4%), injection drug users (7.1%), transgenders and their sex partners (8.8%) (28)

Apart from the high risk group, there is another group of people who play a vital role in the spread of HIV called the "bridging population". This group constitutes the truck drivers and migrant labourers who are usually the clients of sex workers (high risk) bridge the general population and the high risk groups (26).

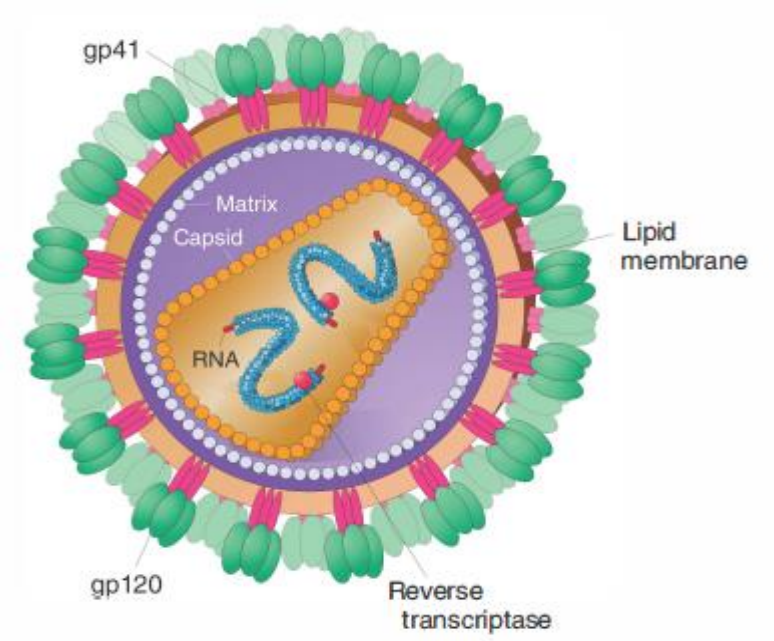
National AIDS control Organisation (NACO) is a part of the Ministry of Health and family welfare that is responsible for policy formulations and implementation for prevention and control of HIV. First AIDS control programme was established in 1992, which aimed at controlling the spread of HIV infection. The current programme is the NACP-IV (2012-2017) which mainly aims at reducing the incidence of new HIV infections by 50% by effective care, support and education (28,31)**.**

3.1.3 Structure of HIV

The mature HIV virion is about 100nm in size and roughly spherical in shape. The rod or the cone shaped inner core contains two copies of single stranded positive sense RNA along with the important enzymes, reverse transcriptase, integrase and protease (3). The lipid bilayer containing various host proteins forms the outer envelope of the virus. The major

envelope proteins are glycoproteins, gp 120 and gp41 which form the surface and transmembrane spikes respectively (32)

Figure 1: Structure of HIV-1



Adapted from Harrison's Principles of Internal Medicine (32)

3.1.4 Genome

The genome of HIV-1 is about 9.7 kb in length. It has three main genes that encode the structural proteins and other accessory genes that encode various proteins that are involved in viral replication (3) The three main structural genes are

- The *gag* gene that encodes the core proteins – p24, p17, p7, p6

- the *pol* gene that encodes various enzymes such as protease (p9), reverse transcriptase (p51/p66) and integrase (p31)
- the *env* gene that codes for the envelope glycoproteins – gp120, gp 41 and gp 160

The *gag-pol-env* genes are flanked by long terminal repeats (LTR) which contains regulatory proteins for gene expression (32)

The additional genes encode for the regulatory (tat, rev) and accessory proteins (nef, vif, vpu, vpr) are important in making the target cell adaptive to augment the viral replication and also to regulate the viral gene expression (32).

3.1.5 Modes of transmission

The three major modes of transmission of HIV are contact with infected body fluids, sexual transmission and mother to child transmission. Transmission largely depends upon the viral load and the duration of exposure to that particular body fluid (6).

Sexual route is the most common mode of transmission of HIV accounting for about 80% of transmission worldwide (33).Certain high risk behaviors such as homosexuality and having multiple sex partners increase the likelihood of HIV transmission. The risk of HIV spread is increased by 300 times when there is concomitant Sexually transmitted infections (STI) caused by *Herpes simplex-2, Hemophilus ducreyi, Treponema pallidum* (6).

Transmission of HIV by blood and blood products has reduced to a large extent after intense screening in transfusion and transplant settings by HIV antibody testing and molecular detection. Injection drug users are the high risk people who acquire infection by

this route. They account for about 7.14% infections in our country as of 2011 especially in the northeastern states (26). Mother to child transmission of HIV can occur at various stages, during pregnancy, labour or through breast feeding which accounts for about 5.4% of HIV infections in our country as of 2011 (34)

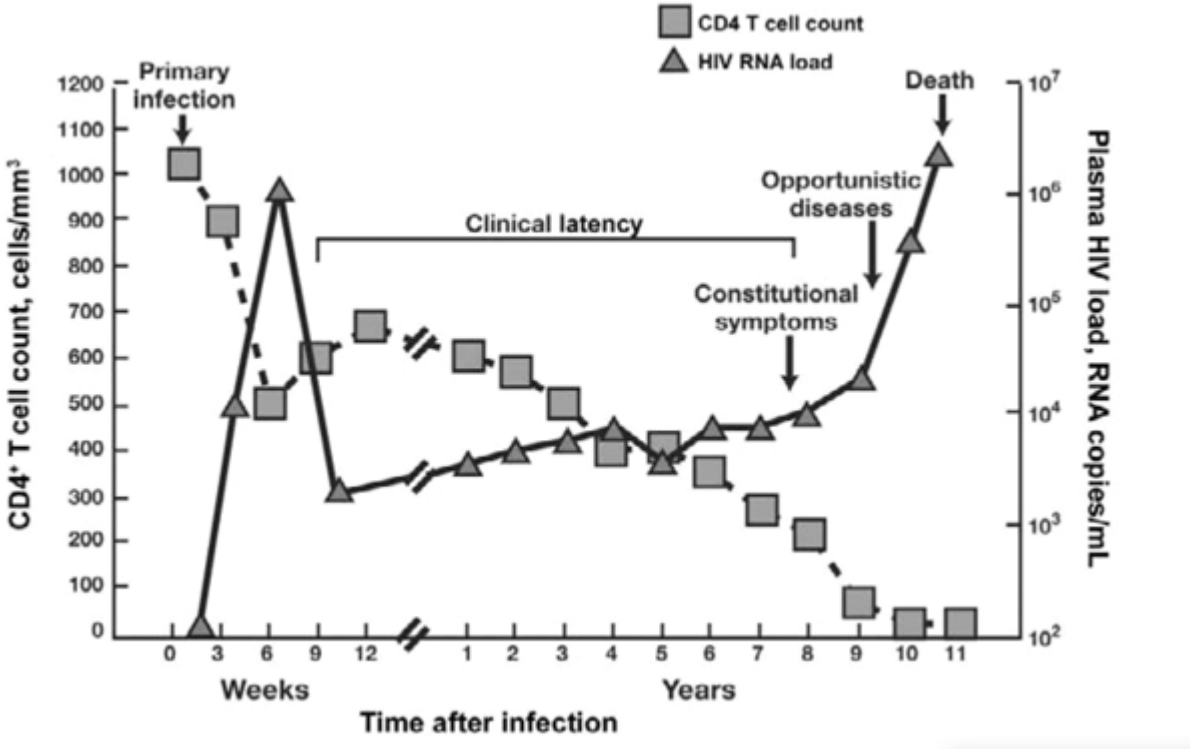
3.1.6 Replication

The primary targets of the virus are the immune cells namely CD4+ T lymphocytes and macrophages. Once the virus enters the body, the surface glycoprotein gp 120 binds to various chemokine receptors on the target cells. CCR5 and CXCR4 are the two main co-receptors found on macrophages and CD4 cells respectively. This binding exposes the transmembrane envelope glycoprotein gp41 leading to fusion of the virion and target cells (33). After penetration, the enzyme machinery of the virus becomes active. The two main enzymes are reverse transcriptase and integrase. The single stranded RNA of the HIV is converted into a double stranded DNA by the reverse transcriptase which is then integrated with the host DNA by the integrase enzyme. Thus the virus replicates along with the host cell's DNA. Following this, transcription occurs to produce mRNA which is then translated into viral proteins in the cytoplasm. The final steps of HIV replication include assembly of the immature virion to the cell surface which leaves the host cell by a process called budding (3,33). The mature virion that is released affects other immune cells thus continuing the process of replication.

3.1.7 Pathogenesis

The telltale sign of HIV infection is the immunosuppression due to progressive depletion of CD4 cells by various direct and indirect mechanisms (6) Once the virus enters the body, there is massive multiplication of the virus in the target immune cells which leads to CD4 cell damage. Despite the host's active immune response, the virus escapes the immunological clearance and leads to release of virions from the destructed cells which are concealed in the regional lymph nodes. This occurs usually within 2-6 weeks when a threshold of replication is reached (35). Thus a primary viremia sets in which is characterized by non-specific symptoms that resembles any viral infection such as fever, lymphadenopathy, rash, sore throat and muscle aches. Once the infection is established, it persists lifelong (36) the primary viremic phase is associated with a high plasma viral load and a transient fall in the CD4 count (35). In proportion to the viral replication, there is profound immune activation by the T cells which leads to release of various proinflammatory cytokines like TNF-α and interleukins. This state in conjunction with the direct viral effects leads to dramatic depletion, impaired production and dysfunction of the CD4 helper cells (37). There exists a period of clinical latency after the primary infection, the duration of which depends upon the individual's immune system. Once the CD4 count falls below 200 cells/µl, the infected individual is prone to get more opportunistic infections (35) and progress to an advanced stage of HIV known as AIDS.

Figure 2: Characteristic course of HIV infection in an untreated individual



Adapted from Pathogenesis of HIV disease, Opportunities for new prevention interventions, Fauci et al, 2007 (37)

3.1.8 Staging of HIV

HIV disease staging is an important tool for making decisions in treatment and monitoring of HIV patients. The staging can be either clinical staging based on the various clinical manifestations with which the patient can present or immunological staging based on CD4 counts. Two major systems of HIV classification are widely used. The WHO classification system which has separate clinical and immunological staging used widely in low resource

setting where CD4 counts may not be available. On the other hand, CDC staging system combines both CD4 counts and clinical staging together (38)

Table 1:WHO clinical staging of HIV (39)

Stages	**Defining illness**
Stage 1	Asymptomatic Persistent generalized lymphadenopathy
Stage 2	Moderate unexplained weight loss (10%) Recurrent respiratory tract infections Herpes zoster Angular chelitis Recurrent oral ulcerations Popular pruritic eruptions Seborrheic dermatitis Fungal nail infections of fingers
Stage 3	Severe weight loss (>10% of presumed or measured body weight) Unexplained chronic diarrhoea for longer than one month Unexplained persistent fever (intermittent or constant for longer than one month)

	Oral candidiasis Oral hairy leukoplakia Pulmonary tuberculosis (TB) diagnosed in last two years Severe presumed bacterial infections (e.g. pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteremia) Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis
Stage 4	**Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations** HIV wasting syndrome Pneumocystis pneumonia Recurrent severe or radiological bacterial pneumonia Chronic herpes simplex infection (orolabial, genital or anorectal of more than one month's duration) Oesophageal candidiasis Extrapulmonary TB Kaposi's sarcoma Central nervous system (CNS) toxoplasmosis HIV encephalopathy

	Conditions where confirmatory diagnostic testing is necessary: Extrapulmonary cryptococcosis including meningitis Disseminated non-tuberculous mycobacteria infection Progressive multifocal leucoencephalopathy (PML) Candida of trachea, bronchi or lungs Cryptosporidiosis Isosporiasis Herpes simplex infection Cytomegalovirus (CMV) infection (retinitis or of an organ other than liver, spleen or lymph nodes) Any disseminated mycosis (e.g. histoplasmosis, coccidiomycosis, penicilliosis) Recurrent non-typhoidal salmonella septicaemia Lymphoma (cerebral or B cell non-Hodgkin) Invasive cervical carcinoma Visceral leishmaniasis

Table 2: CDC staging of HIV (38)

CD4 counts	Clinical stage A	Clinical stage B	Clinical stage C
1. >500 cells/µl	Asymptomatic, acute HIV or Persistent generalized lymphadenopathy (A1, A2, A3)	Symptomatic conditions except A and C (B1, B2, B3)	AIDS defining illnesses (C1,C2,C3)
2. 200-499 cells/µl			
3. <200 cells/µl			

Table 3: CD4 counts and its correlation with opportunistic infections (40).

CD4 count	Opportunistic infections
200 – 500 cells/cu.mm	Candidiasis, Kaposi's sarcoma, *Mycobacterium tuberculosis.*
< 200 cells/cu.mm	*Pneumocystis jirovecii* pneumonia, Histoplsmosis, Progressive multifocal leucoencephalopathy (JC virus)
100 – 199 cells/cu.mm	Toxoplasmosis, Cryptosporidiosis, Cryptococcal infection.
50 – 100 cells/cu.mm	Cytomegalovirus infection
< 50 cells/cu.mm	Mycobacterium avium complex infection.

Opportunistic infections are infections that occurs in individuals with compromised immune system. CDC provides a list of opportunistic infections, which if present, irrespective of the CD4 count, the individual is defined to have AIDS (7).

Apart from the listed infections, any organism can cause significant disease in HIV patients, which is attributed to the profound immunosuppression found in these individuals. The disease can either be due to newer infection or by reactivation of a latent organism (8).

BK virus is one such virus, which belongs to the same family as JC virus (cause of PML in HIV) can cause significant disease in immunocompromised individuals. Case reports of nephropathy, hemorrhagic cystitis, meningoencephalitis have been reported to be caused by BK virus in HIV infected individuals (14)

3.2 BK virus

BK virus is a member of the *Polyomaviridae* family which comprises a group of small, non-enveloped DNA viruses, capable of causing significant nephropathy in immunosuppressed individuals, especially in kidney and bone marrow transplant patients (9).

3.2.1 Discovery

Polyomaviruses (poly – many; oma - tumours) were first reported by Ludwig gross in 1953 who found a filterable agent capable of causing tumours in experimentally infected mice. They named it as murine polyomaviruses (41). Since then till 1960 only three members

were known in this group, the murine or K Polyomavirus, Simian Virus/simian vacuolating virus 40 of rhesus macaques (SV40), found as a contaminant in monkey kidney cells in Salk polio vaccine and the rabbit kidney vacuolating virus (42).

In the next five years, two Polyomaviruses primarily infecting humans, namely JC (John Cunningham) and BK viruses, were described individually by two different groups. JC virus was the first to be identified in 1966 in brain tissue taken from a patient with progressive multifocal leucoencephalopathy (43). Icosahedral shaped virus particles were seen in the oligodendrocytes of this brain tissue and the virus was named after the initials of that patient, John Cunningham (JC) (42). Unlike JC virus, BK virus was an incidental finding in urine of a Sudanese renal transplant patient with ureteric stenosis, collected three and a half months post-transplant, where they actually looked for Cytomegalovirus (9,42). The original strain was known as Gardner's strain, in honor of the Virologist, Dr. Sylvia Gardner, from the virus research laboratory, London, UK. He first observed the viral inclusions under electron microscopy which showed viral particles resembling Papillomavirus. But later when the urine sample was inoculated in African green monkey kidney cells, the cytopathic effect (CPE) seen was different from that produced by Papillomavirus. Thereafter the new virus was identified and named after the patient's initials(BK) (44).These two viruses were the only known members of this family for nearly 40 years while eight new viruses were discovered recently in five years (2007 -2012) (45).

In 2007 two viruses the Karolinska institute (KIPyV) and Washington University virus (WUPyV) were identified in respiratory samples followed by Merkel cell polyomavirus

from a patient diagnosed to have Merkel cell carcinoma affecting the skin in 2008. (15) Another virus called the Malawi polyomavirus (MWPyV) was identified by two separate groups in stool samples of healthy individuals and patients with WHIM syndrome (warts, hypogammaglobulinemia, infections and myelokathexis). The next two polyomaviruses HPyV6 and HPyV7 were isolated from skin and HPyV9 from serum and skin(7) The latest polyomavirus discovered was Trichodysplasia Spinulosa polyomavirus (TSPyV) in 2010 which is associated with a disease producing spiny papules on the skin, especially in immunosuppressed transplant patients.(10) Therefore till date there are 32 known polyomaviruses, with 10 of them capable of infecting humans, but not definitely associated with significant disease(17)

3.2.2 Taxonomy

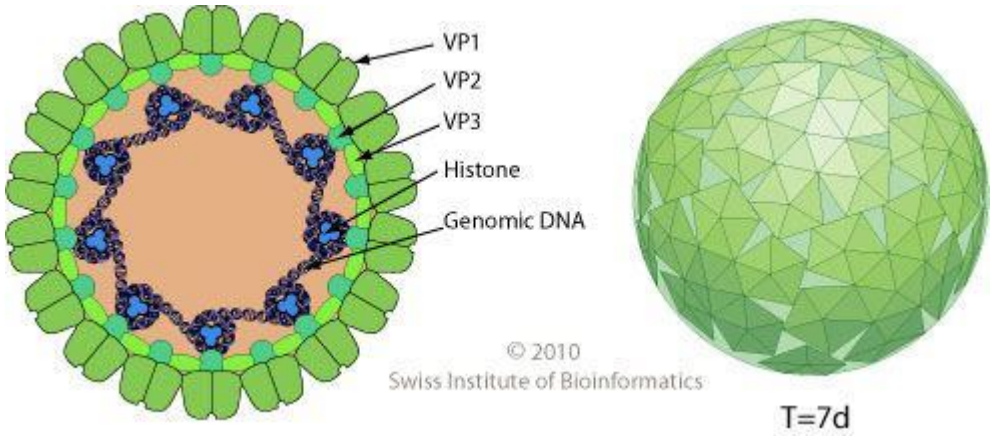
Initially polyomaviruses were placed under *Papavoviridae* along with Papillomaviruses, based on the morphology of the virion, the nature of their genome and the site of replication. But later it was found to have dissimilarity in the genetic organization and evolutionary relationship between the two viruses, making *Polyomaviridae* a separate family (46).

The International Committee on Taxonomy of Viruses revised the taxonomy of the family Polyomaviridae in October 2010 which now contains 3 genera instead of the conventional single genus Polyomavirus. The 3 genera are Orthopolyomavirus, Wukipolyomavirus and Avipolyomavirus, the first two containing mammalian species and the latter containing avian species. The criteria used in creating these taxonomic changes are host specificity,

genetic organization, nucleotide sequence homology over the whole genome (81 – 84% for species). The two important human Polyomaviruses, BK and JC, which show about 60-70% sequence homology are under the genus Orthopolyomavirus (47).

3.2.3 Structure and genome

Figure 3: Structure of BK virus



Adapted from viralzon.expasy.org

BK virus is a small, non-enveloped, DNA virus measuring about 40 – 45nm in size. The viral capsid is of icosahedral symmetry which consists of 72 capsomeres enclosing covalently closed circular double stranded DNA (48).

The genome of the virus is about five kilobasepairs in length encased around the host cell derived histones. The genome is divided into 3 functional regions namely the early gene region, late gene region and the non-coding control region (NCCR).

The early gene region codes for the large T antigen (LTag) and the small T antigen (sTag) whose function is to enhance the viral replication and transformation (10). The mechanism of the large T antigen facilitating viral replication is by its binding to the tumour suppressor proteins, the retinoblastoma (Rb) and p53, thus enhancing the host cell entry into the cell cycle (49). The other proposed functions of the large T antigen includes helicase activity which enables unwinding the strands of DNA thus opening up the origin of replication. It also facilitates the expression of host cell enzymatic system such as DNA polymerase or alpha primase complex thus playing an important role in the viral replication (14).

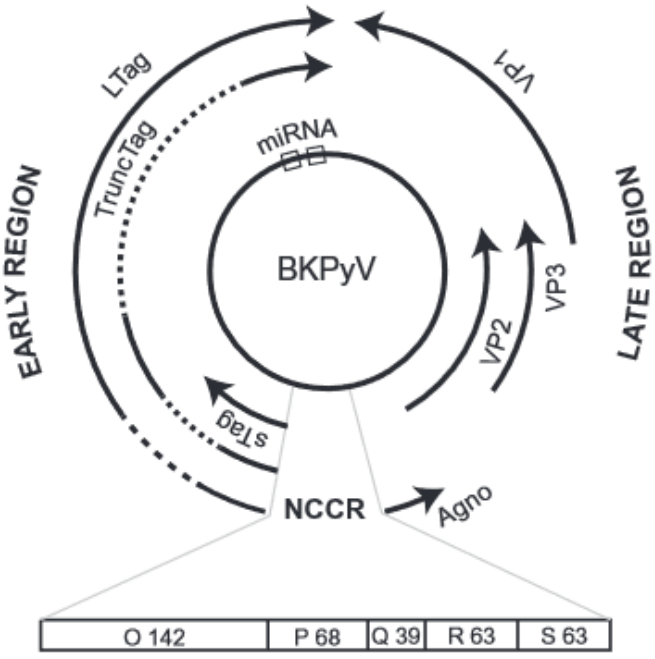
The late gene region codes for the capsid proteins namely VP1, VP2 and VP3 responsible for the capsid production and assembly. The VP2 and VP3 share coding sequences in the same reading frame whereas VP1 gene is translated in a separate reading frame. In addition, the late region codes for a non-structural protein called the agnoprotein. The function of this small cytoplasmic agnoprotein remains unclear with controversial evidence on the release of infectious progeny as its main function (50)(51).

The NCCR region contains the origin of replication (ori) and the regulatory regions which contain the tandem repeats designating the enhancer elements responsible for early and late viral transcription (44). This region is conventionally divided into four blocks as O, P, Q, R, and S. The O block contains the origin of replication and transcription binding sites while the rest of the blocks have the promoters and the enhancers of transcription control of early and late gene regions.

The division of base pairs among the various blocks of NCCR (O142bp,P63bp, Q39bp, R63bp, S63bp) has arbitrarily been done such that an archetypal ww-NCCR strain has been

put forth (52). Any variations in this archetypal NCCR region in the form of point mutations, deletions or duplication is considered as the rearranged rr-NCCR. These rearrangements in the NCCR region have shown to influence on the cell permissivity and rate of viral replication. This has been well documented in JC virus associated PML (Progressive Multifocal Leucoencephalopathy) patients where pathologic lesions in brain were found to be severe in cases with NCCR rearrangements (44). In case of BKV nephropathy, NCCR rearrangements have been associated with high BK viral load, increased likelihood of getting histologically evident disease and a higher degree of cytopathological changes (53).

Figure 4: Schematic diagram of BKV genome.



Adapted from The human polyomaviruses, Virological background and its clinical implications, Rinaldo et al, 2013 (54)

3.2.4 Genetic diversity

Based on the VP1 genetic diversity, BKV has been classified into 4 major subtypes as I, II, III, and IV. The BKV subtype I is found to be the most prevalent type ranging about 46-82% worldwide. The second most common subtype is the subtype IV, being prevalent in Asia and Europe especially reported from the Northeast Asia. The other two subtypes are rarely reported with the frequency ranging from 0 to 6-9% (11,55).

The subtypes I and IV are further divided into subgroups. Subgroups of subtype I are Ia,Ib1,Ib2 and Ic being commonly found in Africa, South East Asia, Europe and North East Asia respectively and those of subtype IV are IVa1 commonly reported in strains from South East Asia, IVb1 from Korea, IVb2 from Japan and IVc from China (55,56).

Four genotypes I, II, III, and IV have also been described based on NCCR sequences. But the geographic distribution of these NCCR based genotypes have not been well studied (12).

3.2.5 Epidemiology

Humans are the well-known host for BK virus and serological evidence has been considered to be the best source to study its prevalence (44,57), which was done most commonly by Hemagglutination inhibition(HI) assay detecting IgM and IgG against capsid antigen followed by complement fixation, indirect immunofluorescence and immunoelectroosmophoresis which was then replaced by ELISA using recombinant antigens and more recently with virus like particles (58).

Infection with BK virus occurs during childhood via respiratory route, preferably after the disappearance of maternal antibodies. The initial infection is usually asymptomatic or with

a mild flu like upper respiratory illness with fever (23,59). After the primary infection, the virus has the ability to be latent for years in various sites, the most common being the kidney, especially in the renal tubular cells and urothelial cells with the detection rate of about 50% in healthy kidneys. The second most frequent site of latency is the peripheral blood mononuclear cells which is followed by brain (13,14). Seroconversion has been reported up to 50-60% as early as 10 years of life reaching around 90 % in adults. There are reports of BKV DNA detection in tonsillar tissue in children who presented with upper respiratory illness (9,15). By large there is no significant gender difference in the BK seroprevalence even though rare reports of male predominance have been found (60).

3.2.6 Replication

The replication of BK virus is a complex process and is poorly understood as there are only few evidence based studies describing the various aspects of this replication.

The early phase of replication starts with attachment of the virus by VP1 to various cellular receptors such as ganglioside GD1b and GT1b containing alpha 2-8 linked sialic acid residues. Other receptor which has a doubtful role in attachment is the N-linked glycoprotein with alpha 2-3 linked sialic acid residues (61). After the first step of VP1 binding, the virus is internalized by caveolae mediated endocytosis. Caveolae (caveolin containing lipid rafts) are plasma membrane folds which are involved in various transport mechanisms across the cell (62).

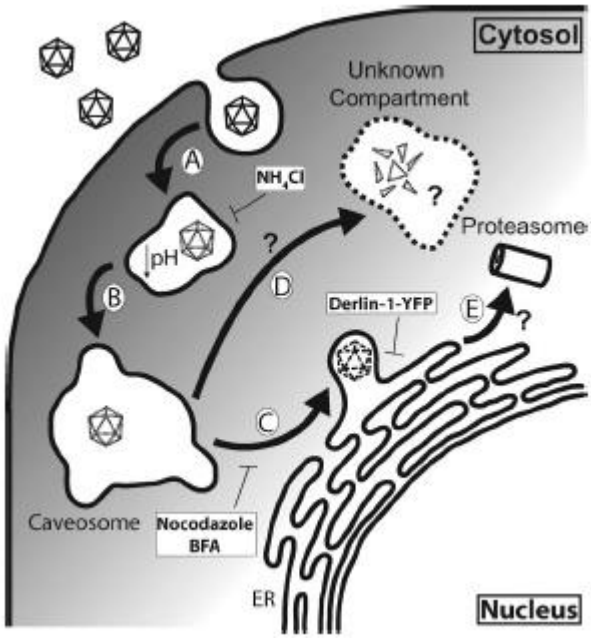
Following internalization, the virus enters the endoplasmic reticulum which is facilitated by an intact microtubule system and actin filaments within 6 to 8 hrs of infection (63). As BK virus is a DNA virus the replication takes place in the nucleus. The transport of this

virus from the endoplasmic reticulum to the nucleus occurs by an unknown mechanism. But it has been hypothesized that a family of proteins situated in the membrane of the endoplasmic reticulum (ER), called the Derlin family is responsible for this ER to nucleus transport of the virus. These proteins are actually involved in the transport of misfolded proteins from ER to the cytosol for proteosomal degradation (61). It has also been proposed that VP2 has a role in this transport by producing various signals localizing the virus to the nucleus (64).

In the nucleus, replication process continues with transcription of the early gene region producing the large T and the small t antigens where the LTag, inhibits the early gene expression subsequently enhancing the late gene transcription. This along with other proposed functions makes the surrounding receptive for viral replication. The small t antigen also has a considerable but not a vital role, in enhancing the lytic replication of the virus (65).

The late gene region translates to produce the capsid proteins in the cytoplasm which are then brought back to the nucleus for viral assembly. Here is the role of agnoprotein which is present in the perinuclear region which is proposed to do the process of assembly. Once there is massive replication and production of virions, the affected cell lyses to release the progeny virus in the environment which will further infect new cells thus making the infection productive (65)

Figure 5: Initial steps of BK virus replication.



Adapted from Early events during BK virus entry and assembly, Jiang et al, 2009 (61)

3.2.7 Immunity

The immune response elicited by the host towards a viral infection is of primary importance in limiting the initial viral replication and the host carrier state. The innate immune response comes into play even before the specific antibody response and HLA restricted T cell response occurs. Innate response activated through TLR-3 has a role in controlling the BKV infection and eliciting an inflammatory response against BKV(66) Humoral immune response in BK infection is elicited by the development of neutralizing antibodies which has very limited role in restricting the BKV replication and in development of significant nephropathy (66–68).

Cell mediated immune response in viral infections is exhibited in various ways like cytotoxic activity by CD8 T lymphocytes, release of proinflammatory cytokines which initiates a massive recruitment of lymphocytes and direct antiviral activity thereby playing a potential role in limiting the viral replication (69).

In case of BK virus infection, the cellular immunity acts on various antigenic targets mainly the capsid proteins (VP1, VP2, VP3), and T antigens (large and small) eliciting an effective immune response especially involving CD4 T cells (91%) than CD8 T cells (33%) (68). The CD4 cells recruited are polyfunctional. These cells have a large multiplicative capacity and release a wide range of cytokines especially interferons thereby effectively limiting the viral replication. This multifunctionality of CD4 cells is seen better in young age especially between 20 to 30 years thus having an age preponderance similar to antibody response to BK viral infection (70). In immunosuppressed individuals, especially when the cellular immunity is depleted as in case of HIV infection, there is reactivation of the latent BK virus. This along with its unrestricted replication cause viral shedding in urine and blood thus producing a significant disease (67,68).

3.2.8 Pathogenesis

3.2.8.1 Routes of transmission

There are various proposed modes of transmission such as transfusion of blood and blood products, organ transplantation, feco oral, urino oral, contact with any other body fluids. As the primary infection occurs through respiratory route producing a mild respiratory illness, the major mode of transmission is by aerosols or fomites (9,15,16). Sexual mode of transmission has been evidenced by detection of BK virus in genital tissues (57%) and

semen (95%) (15,23). Transplacental route has been documented as a potential mode of transmission after detection of nucleic acid in aborted fetal material and placental tissues by molecular techniques and demonstration of IgM antibodies in cord blood (16,23).

3.2.8.2 Latency

After the primary infection, BK virus is capable of being latent in forms of low replicating or a non-replicating dormant state in various cells over a period of time without causing any significant morbidity.(23)This latent state is evidenced by asymptomatic shedding of the virus in urine of healthy individuals(10). The most likely sites of latency for BK virus are the kidneys and ureters proving the urotheliotropic nature of the virus. BK virus has been reported in 30 – 50% of healthy kidneys and 40% in ureteric epithelial cells. The second most common site of BK virus persistence is the brain, found both in normal and tumour tissues but in considerably low levels than found in urothelial cells. The next common site following brain is the peripheral blood cells and spleen (15,23).

3.2.8.3 Reactivation

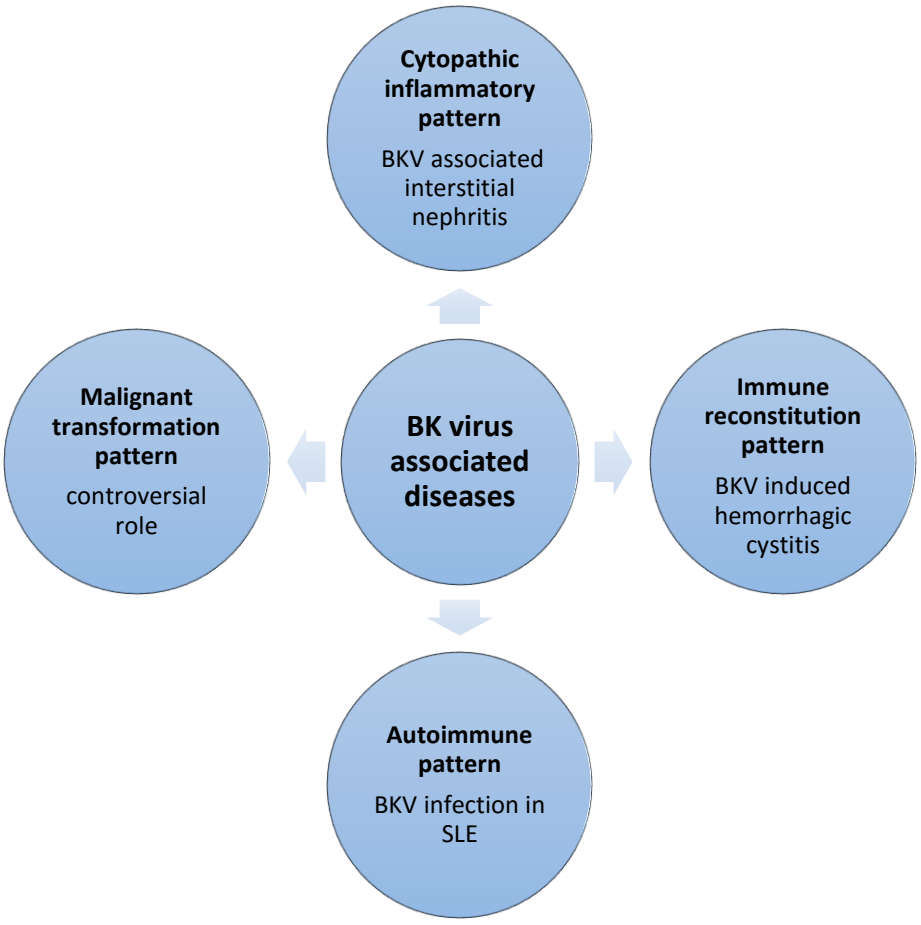
Human Polyomaviruses are capable of getting reactivated from the latent non replicative state to a high replicative state producing lytic infection. When the host immune system is compromised, virus reactivation occurs and when there is a high viral load, viral shedding in the urine and blood occurs, subsequently leading to significant disease (15). The consequence of BKV reactivation depends on the degree of immunosuppression.

It ranges from asymptomatic viruria without significant morbidity in mild immunosuppressive states like pregnancy, old age, diabetes mellitus to severe disease occurring in profound immunosuppressive conditions such as bone marrow

transplantation, solid organ transplantation especially kidneys, Acquired immunodeficiency syndrome (AIDS), lymphoproliferative states, autoimmune conditions like systemic lupus erythematosus (15,23).

Different patterns of pathogenic mechanisms have been explained for BK virus induced diseases(16)

Figure 6: Different pathogenic mechanisms of BK virus



Adapted from Polyomavirus BK, Hirsch HH and Steiger J, 2003 (16)

3.2.8.4 In transplant settings:

As kidneys are the main site of latency for BK virus, their reactivation cause significant nephropathy in transplantation settings as immunosuppression in the form of chemotherapy sets in here. About 60% of renal transplant patients and 10-50% of hematopoietic stem cell transplant patients have been reported to have BK virus associated nephropathy (BKVAN) and late onset hemorrhagic cystitis respectively (15,48).

There are various other determinants that influence the BK virus reactivation in transplant patients, such as choice and duration of immunosuppressive therapy, degree of HLA matching, BK virus genotype (71,72).

In renal transplantation, with the advent of third generation immunosuppressive agents such as tacrolimus and mycophenolate mofetil, the incidence of BK virus associated interstitial nephritis has increased by about 13 fold. This proves that the choice of immunosuppressive regimen plays a vital role in disease pathogenesis. This combination of tacrolimus and mycophenolate mofetil has been hypothesized to increase the BK virus replication by unknown mechanisms (71) Other factors which determine the BKV disease in renal transplant recipients are(73)

- Deceased donor transplant (increased cold ischemia time)
- BKV seropositivity of donor
- BKV seronegative pediatric recipients
- Use of ureteric stents
- Acute graft rejection

- Retransplantation for a graft loss due to polyomavirus induced nephropathy

Following reactivation in the proximal tubular epithelial cells (primary latent site), there is massive multiplication of the reactivated virus resulting in lytic destruction of the affected cell, with spread infection to the adjacent epithelial cells. This along with fluid accumulation in the interstitial compartment, results in severe interstitial nephritis finally leading to irreversible dysfunction of renal tubular cells due to fibrosis and atrophy (23,71) Apart from interstitial nephritis, BK virus has been implicated in ureteral stenosis accounting for about 8% in patients with allograft dysfunction (16).

In bone marrow transplantation, late onset hemorrhagic cystitis has been highly associated with BK virus reactivation, accounting for about 10-29% in these patients (17). Hemorrhagic cystitis is a condition frequently seen with allogeneic hematopoietic stem cell transplantation than with solid organ transplantation. This can be characterized by features of cystitis with varying grades of hematuria. It can be an early onset disease, which is usually due to non-viral factors such as the use of conditioning immunosuppressive regimen containing cyclophosphamide/busulfan and total body irradiation which cause direct toxic effects to the urinary bladder mucosa causing significant morbidity within one week of transplantation (16,17).

The late onset disease, which is of viral origin, usually occurs after one week to two months of transplantation, (16,17)with BK virus being the most common cause even though other determinants play a role in the pathogenesis of the disease such as Co-viral infections like cytomegalovirus and adenovirus, Graft versus host reaction and advanced age (74).

It has been proposed that the pretransplant preparative therapy reactivates the dormant BK virus in the tubular cells, leading to its massive multiplication and cytopathic effects. Indirect cellular damage occurs due to the regenerating immune system in the post-transplant period which targets the abundant viral antigens present due to the unrestricted proliferation of the virus, producing severe inflammation and damage to the bladder mucosa. But the hypothesis of immune reconstitution mechanism does not hold well in lymphopenic patients, proving the role of other unknown factors in the pathogenesis of BK virus induced hemorrhagic cystitis (71).

3.2.8.5 In HIV infection

As HIV causes profound immunosuppression by invading the CD4 helper T cells which are the group of cells responsible for restricting BK viral replication, this subset of patients are prone to get BKV associated diseases. The role of BK virus in HIV infection is not well known even though case reports of varied clinical spectrum have been reported. This includes hemorrhagic cystitis and BKV associated nephropathy followed by sporadic reports of meningoencephalitis, retinitis and pneumonia (14,16).

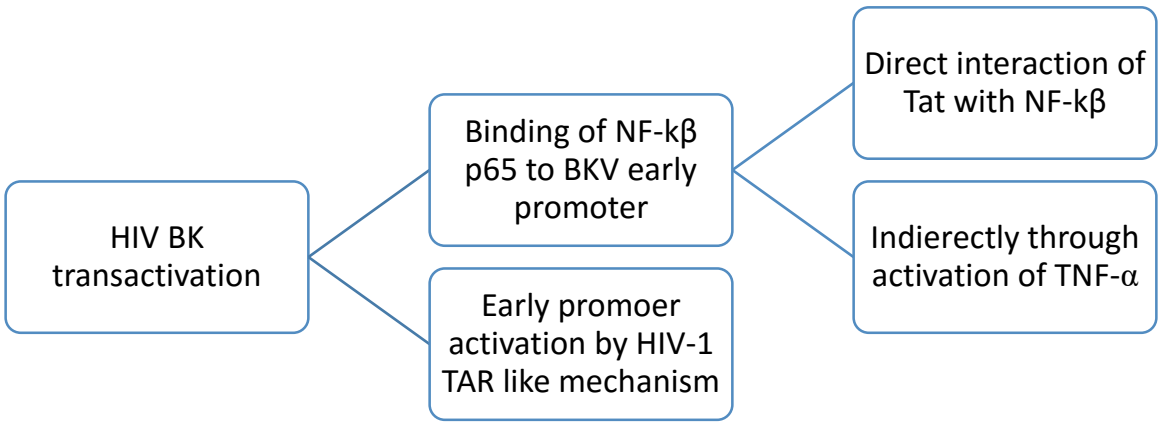
HIV-1 is capable of transactivating many viruses thus augmenting their pathogenicity by enhancing their viral replication by its various gene products. The most important gene is the Tat gene (Transcriptional transactivator) which codes for one of the regulatory proteins of HIV involved in the transcription elongation from HIV-1 long terminal repeat.

Tat gene exerts its effects on replication of other viruses in two different ways by either directly acting on the viral promoters to activate their transcription or indirectly by

affecting the transcription of various cytokines involved in the genomic transcription of various viruses through NF-kβ Eg: TNF-α in JC virus transcription (75).

In case of HIV and BK virus co-infection, the Tat protein secreted by the affected cells transactivates the BKV early promoter gene by different mechanisms as shown below, resulting in its efficient transcription and thus increased viral replication enhancing their pathogenicity(76)

Figure 7: HIV BK transactivation mechanisms (76)



TAR – Trans activation response elements

Conversely, the early gene region protein of BK virus, the large T antigen (LTag) stimulates the HIV-1 large terminal repeat transcription thus having a reciprocal activation between the two viruses (77).

3.2.8.6 In carcinogenesis

The role of BK virus in human carcinogenesis is still controversial even though there are well demonstrated evidences for its oncogenic potential in animal models. BK virus infected hamster models have shown a variety of tumors with ependymal tumors, neuroblastoma, choroid plexus papilloma, osteosarcoma being the most common tumors (78). As Bk virus is ubiquitous and can be found even in normal tissues, there are criteria for proving the causative role of this kind of controversial oncogenic viruses in carcinogenesis. They are (79)

- Constant presence of viral DNA in the tumor cells
- Neoplastic transformation of cells after its transfection with the suspected viral genomic fragments
- Demonstration of malignant phenotype of the transformed cells which is due to the specific functions expressed by the viral genome
- Clinical and epidemiological evidence that the suspected viral infection is a predominant risk factor for the tumor development.

The oncogenic potential of BK virus is proved to be due to the expression of the early region proteins, the large T (TAg) and the small t (tAg) antigens alone or in combination with other oncogenes like *ras* and *myc* genes (80,81).

The TAg plays a vital role by binding to various families of cellular tumour suppressor genes such as p53 and retinoblastoma gene. p53, is an important housekeeper gene which has an effective check on the G1 to S transition of cell cycle especially when there is a DNA damage. The retinoblastoma susceptibility proteins pRb and its family members

p130, p107 whose primary function is to regulate the rate of cellular growth and to induce growth arrest when needed (81)

The TAg binding to these proteins, functionally inactivate them causing an unchecked multiplication of cells subsequently leading to tumour formation. It has also been postulated that the TAg interaction with the growth regulatory proteins has an effect on the rate of cell proliferation. However, for complete malignant transformation of cells, a massive expression of the early gene region is required that effectively inactivates the tumour suppressor genes(78,81)

The tAg on the other hand has a supplementary role in oncogenesis of BK virus. This acts by binding to a protein phosphatase 2A (PP2A), which is a heterotrimeric serine/threonine phosphatase which modulates phosphorylation signals generated by protein kinases. Recently, this enzyme has been considered to be a tumour suppressor gene in various carcinomas.

The tAg binds to its catalytic and regulatory site of this enzyme, leading to activation of Mitogen Activated Protein kinase (MAPK) pathway and down regulation of Wnt/β-catenin pathway thus causing unchecked cell proliferation(78,80)

3.2.9 Pathology

3.2.9.1 BKV associated nephropthy (BKVAN)

As renal biopsy forms the gold standard for the diagnosis of BKVAN, pathological examination forms one of the mainstay of diagnosis of this condition (59).

Macroscopically, a BKV affected kidney looks slightly shrunken with ill-defined corticomedullary junction showing patchy fibrosis of the medulla and thin sclerosing cortex with fibrotic scars of varying sizes (82,83)

Table 4: Histologic patterns of BKV associated nephropathy

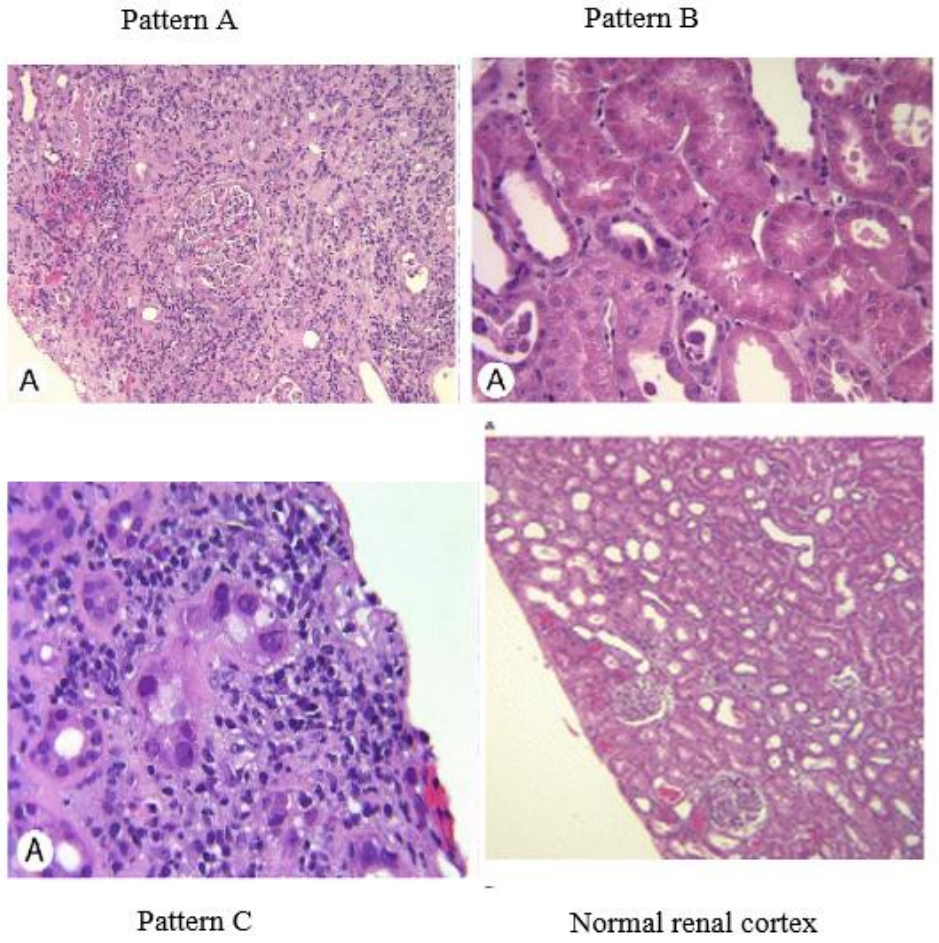
Patterns	Histological features
A (early)	Viral cytopathic changes only with minimal or no tubular inflammation/atrophy
B (intermediate)	Viral cytopathic changes with varying degrees of focal/multifocal tubular inflammation/atrophy/interstitial fibrosis. Can be subdivided according to the extent of tubular involvement and fibrosis B1 - < 25% B2 – 26-50% B3 - >50%
C (end stage PVN)	Minimal cytopathic changes with massive tubular atrophy interstitial fibrosis in a background of chronic inflammation

Histologically, the disease is characterized by the presence of viral cytopathic changes in the tubular epithelial cells, which is the site of active viral multiplication. The affected cells show extensive necrosis with intranuclear basophilic inclusions giving a ground glass appearance. These cells are typically called as “decoy cells”. This feature

along with interstitial fibrosis gives an overall picture of tubulointerstitial nephritis(83,84)

Based on the presence of cytopathic changes and degree of tubule loss, there are three main histological patterns A (early), B (intermediate), C (late) predicting the outcome of the disease.(84,85)

Figure 8: Histologic patterns of BKV nephropathy



Adapted from Histologic patterns of Polyomavirus nephropathy, correlation with graft outcome and viral load, Drachenberg et al, 2004 and Polyomavirus disease in renal transplantation: Review of pathological findings and diagnostic methods Drachenberg et al, 2007

This virus induced tubular cell damage has a clinical and diagnostic significance. The clinical implication is that extensive tubular damage finally disrupt the basement membrane leading to viremia and the degree of inflammation seen with tubular injury may mimic an acute rejection in transplant settings. For diagnosis, the shedding of necrosed cells with virions are used for the cytological and molecular studies (85).

Other histological findings which can be seen are glomerular crescents, ischemic glomerulopathy and tubular microcalcifcations (84).

3.2.9.2 Brain and lung

Less commonly, case reports have shown that BK virus has been implicated in causing sub-acute meningoencephalitis and desquamating pneumonitis as these areas are rare sites of viral latency.

Microscopic examination of brain tissue showed thickened fibrotic leptomeninges with mild astrocytosis and predominant mononuclear infiltrate.

Main histological feature of BK pneumonitis lung was fibrosis with aggregates of pneumocytes inside the alveoli (83).

3.2.10 Clinical features

Primary infection with BK occurs in childhood, which presents as a mild respiratory illness, following which a long asymptomatic latent state occurs in the renal tubular cells. Reactivation of the virus in healthy individuals is usually rare, but asymptomatic viruria occurs in about 5-7% without viremia (17).

In immunocompromised states, there is reactivation and rapid multiplication of the virus. This causes significant morbidity manifesting in three different scenarios as BKV associated nephropathy, hemorrhagic cystitis and ureteric stenosis.(17)

3.2.10.1 BKV associated nephropathy

BKV nephropathy is common in renal transplant patients, with reactivation occurring in 30-50% of patients after 3 months of transplantation. Viruria occurs in 80% of renal transplant patients with 5-10% progressing to nephropathy (86). This condition is rare in immunocompromised states other than renal transplantation, thus emphasizing the importance of various factors associated with transplantation in development of nephropathy (54)

Patients present around 10-13 months post-transplant, with deterioration of renal function evidenced by the rise in creatinine (86). Typically these individuals lack features of viral infection such as fever or decrease in blood cell counts in spite of the high BK viral loads in their urine and plasma (87). The disease starts with viruria followed by viral shedding in plasma. Subsequently, BK virus will be found in renal tissue, leading to allograft failure with rates as high as 50-80% (86).

3.2.10.2 Hemorrhagic cystitis

BK virus associated late onset hemorrhagic cystitis occurs in about 5-10% of the allogeneic bone marrow transplant patients (54). Clinically patients present between 25 to 50 days post-transplant, with hematuria and other symptoms of urinary tract infection such as dysuria, increased frequency and supra pubic pain. Renal failure occurs due to obstruction of the urinary tract with large blood clots (87).

Grading of hemorrhagic cystitis

There are 4 grades of hemorrhagic cystitis based on the clinical manifestations of the disease. They are (88)

Table 5: Grades of BKV associated hemorrhagic cystitis

Grades	Clinical manifestations
I	Microscopic hematuria
II	Macroscopic hematuria
III	Macroscopic hematuria with blood clots
IV	Deterioration of renal function due to urinary obstruction which need intervention

3.2.10.3 Ureteric stenosis

There are reports of ureteric stenosis due to BK virus in both renal and allogeneic bone marrow transplant recipients. This occurs approximately within one year of transplantation, seen more with patients who shed the virus in blood than aviremic patients (23). Stenosis can be reversible or irreversible. Reversible obstruction occurs secondary to obstruction of the urinary tract due to blood clots in hemorrhagic cystitis (89). Irreversible obstruction happens due to fibrosis following excessive inflammation and urothelial damage in BKV nephropathy (54).

3.2.11 Diagnosis

Since its discovery in 1970, serology and urine cytology for decoy cells were used as the main methods of diagnosis for BKVAN till 1985 (90). Various serological tests were widely to detect both IgM and IgG antibodies against BK virus (58). As the primary infection occurs in early childhood, neutralizing antibodies were found in about 50-60% of children aged <10 years. The seroconversion rates reach up to 90% in adults. These antibodies are not protective against the disease and just indicate the previous exposure to BK virus (91). Thus, other diagnostic methods were adapted for the detection of BK virus. The first case diagnosed by histopathological examination (now the gold standard method) was reported from the University of Pittsburgh in 1993. The current modes of diagnosis of BKV associated nephropathy are urine cytology for decoy cells, molecular methods such as quantitative PCR and histopathological examination of biopsied tissue (90).

3.2.11.1 Urine cytology

This was one of the earliest methods used for the diagnosis of BK virus in transplant patients. The importance of cytological examination for diagnosis of various diseases was first described by George Papaniculou in 1945. Later the procedure was used for diagnosis of Polyomavirus infection by Koss et al. They called these cells as “Decoy cells” to discrimnate it from malignant cells (92)

This is now used as a screening tool to monitor BKV viruria in transplant patients (8). When cytospin smears of urine from BKV infected individuals are stained with Papaniculou stain, characteristic cells with viral inclusions are seen. These are the virus infected epithelial cells which have large, homogenous and basophilic intranuclear

inclusion giving a ground glass appearance. They have a high nuclear cytoplasmic ratio with peripheral rim of condensed chromatin (93)

There are four varieties of decoy cells described. They are (92)

Table 6: Types of decoy cells

Type	Description
Type 1 (classic variant)	Large, homogenous and basophilic intranuclear inclusion giving a ground glass appearance
Type 2 (Cytomegalovirus like variant)	Granular inclusion with a halo around it
Type 3	Cells with multiple nuclei and granular chromatin
Type 4	Cells with vesicular nuclei and condensed chromatin

Thus detection of decoy cells has a reliable sensitivity of about 84 - 100% in detecting apparent BKVAN, but a very low positive predictive value of 29% thus requiring further tests to confirm the disease (72). Even though decoy cells are predominantly found in BK virus infections, these are not pathognomonic for this infection. It can also be found in JC and adenovirus infections (8). Therefore, the cause of decoy cells must be confirmed by molecular techniques.

3.2.11.2 Molecular assays

Quantitative polymerase chain reaction (PCR) on blood and urine is considered to be more sensitive than urine cytology in BK virus detection (8). Viral shedding in urine has lower disease specificity as increased levels of viruria (20-60%) is usually observed in HIV infected individuals, even without significant nephropathy (54). Thus demonstration of the virus in blood/plasma is considered to be the better indicator of disease and thus viral replication. Quantitative BKV DNA PCR has a sensitivity of 100% and specificity of 88% in diagnosing BKVAN (72). Higher viral loads has been found to be associated with increased tendency to get BKV nephritis (72). However, the cut off values for quantitation of BK shedding in urine and blood has not been defined in HIV infected individuals (8). In case of renal transplant patients, PCR is used as an important tool for monitoring as 15-30% of patients have detectable viral loads in their first year of transplantation (72). But threshold values overlap between active and resolved nephropathy thus making histological examination the gold standard for BKV diagnosis (84).

3.2.11.3 Histopathology

Histopathological examination of renal tissue forms the mainstay of diagnosis in BKV associated disease. As biopsy gives an indiscriminate information about the extent of renal damage and thus disease progression, this is considered to be the gold standard method of diagnosis (59). At least two core biopsies which include the medullary tissue is required for examination as the viral cytopathic changes can be localized (84). Typical viral cytopathic changes such as amorphous, basophilic intranuclear inclusions which gives a ground glass appearance can be observed in the urothelial and tubular epithelial cells (85).

Similar cytopathic changes can be seen in JCV, SV 40 and adenovirus infections which can be differentiated only by BKV specific immunohistochemistry (84). The damaged tubules show a spectrum of changes from mild inflammatory changes to necrosis and atrophy. Loss of renal tubules leads to massive parenchymal scarring and this along with fibrosis gives a picture of tubulointerstitial nephritis (85). The extent of tubular damage in conjunction with the cytopathic changes form the basis for the histological patterns described. (Patterns A, B & C) (16,59). In transplant settings, this histological grading is used as a prognostic marker as this represents the stage of the disease and graft outcome (85).

3.2.12 Treatment

Till date, there is no specific anti BK therapy. Various management approaches have been tried which all emphasize the basic keystone, reduction of immunosuppression (71). With the widespread use of new and effective immunosuppressive agents, the occurrence of BK virus associated diseases has increased in the recent years (22). The offending agents, tacrolimus and mycophenolate mofetil when given in combination with other immunosuppressive drugs make the host unable to mount an effective antiviral immune response. Thus the hallmark of treating BKV disease would be either reduction or discontinuation of the immunosuppressive therapy (22,71). This is the only approach which has definite role in the management of BK patients. Even this approach becomes dubious in the scenario of concomitant BKV disease and acute graft rejection, as reduction of immunosuppression causes rapid graft rejection and poor graft survival (22). But, as graft

rejection settles down rapidly than BKV clearance treatment of graft rejection should be the first priority (71)

The other modalities of treatment which have doubtful role are the use of Leflunomide, Cidofovir intravenous immunoglobulins, fluoroquinolones and statins (22,71,94). Leflunomide is an immunomodulatory and anti-inflammatory drug which inhibits pyrimidine synthesis and tyrosine kinase activity. This drug has an in vitro activity on BK virus and has proved to reduce the incidence of graft loss when used along with modification of immunosuppressive therapy (71)

The antiviral agent, Cidofovir which is primarily used against CMV retinitis has been tried in the management of BKV nephropathy. In vitro studies have shown that this drug acts by inhibiting the BK virus replication through unknown mechanisms. Because of its potent nephrotoxicity, its proposed use in BKV disease is controversial (94). Even though various studies state the use of this drug in BKV associated diseases, the lack of large randomized controlled trials limits its use in these settings (95,96)

Intravenous immunoglobulin therapy is proposed to have neutralizing antibodies against BKV, but its role is highly controversial (94).

Systematic reviews have shown that no added benefits have been obtained with adjunctive therapies such as Leflunomide or Cidofovir. Thus to conclude, with the lack of specific anti BK therapy and debatable role of other modalities, only reduction of immunosuppression is the definitive approach for the management of BKV associated diseases

4. MATERIALS AND METHODS

This study was done at the Department of Clinical Virology, Christian Medical College and Hospital (CMCH), Vellore The study was approved by the Institutional Review Board of the institution (Reference no IRB Min No. 8982 dated 04.08.2014)

4.1 Study subjects

4.1.1 HIV-1 infected individuals – treatment naive

ART naive HIV-1 infected individuals who had come for routine CD4+ T cell estimation and or HIV-1 viral load were included in this study. As part of the patient management, these study participants were regularly visiting the clinics of our hospital departments like, Departments of Infectious disease, Internal medicine and Dermatology. The study was explained to all the participating individuals and was recruited in the study only after getting a written informed consent. This cross sectional study was done during a period of 1 year (August 2014 – August 2015)

4.1.1.1 Inclusion criteria:

1. Adults >18yrs of age
2. HIV-1 infected individuals (serologically confirmed by WHO/NACO strategy III))
3. Treatment naive
4. Individuals willing to give both blood and urine samples

4.1.1.2 Exclusion criteria:

1. HIV-1 infected individuals on treatment
2. Individuals not giving consent for collection of samples
3. Individuals < 18years of age

4. Pregnant women

5. HIV-2 infected individuals

4.1.2 HIV-1 infected individuals – post ART

Urine and whole blood samples were collected from a proportion of HIV-1 infected individuals after a minimum of 3 -6 months of ART.

4.1.3 Healthy controls

Urine and whole blood samples were collected from healthy individuals (considered to be HIV negative) to look at the frequency of BK virus in this population as this is an opportunistic virus.

4.2 Sample size:

As per the frequency of BK viruria in HIV infected individuals published in Nali et al 2012 (97), the p value (prevalence) is taken to be 18 ,q (100-p) is 82, and d the degree of precision taken as 6 applying in the formula,

$$4pq/ d^2$$

$$4*18.7*81.3 / 36 = 169$$

Even though the calculated sample size was 169, urine and blood samples were collected from 187 consecutive treatment naïve HIV-1 infected individuals who attend the Infectious Disease clinic, CMC and referred to the Department of Clinical Virology for CD4+ T cell

testing and or HIV-1 viral load. The samples were collected from those who gave consent for the study.

Since we collected 187 patient samples, 93 (1 control for 2 patients) healthy individuals were recruited in the study.

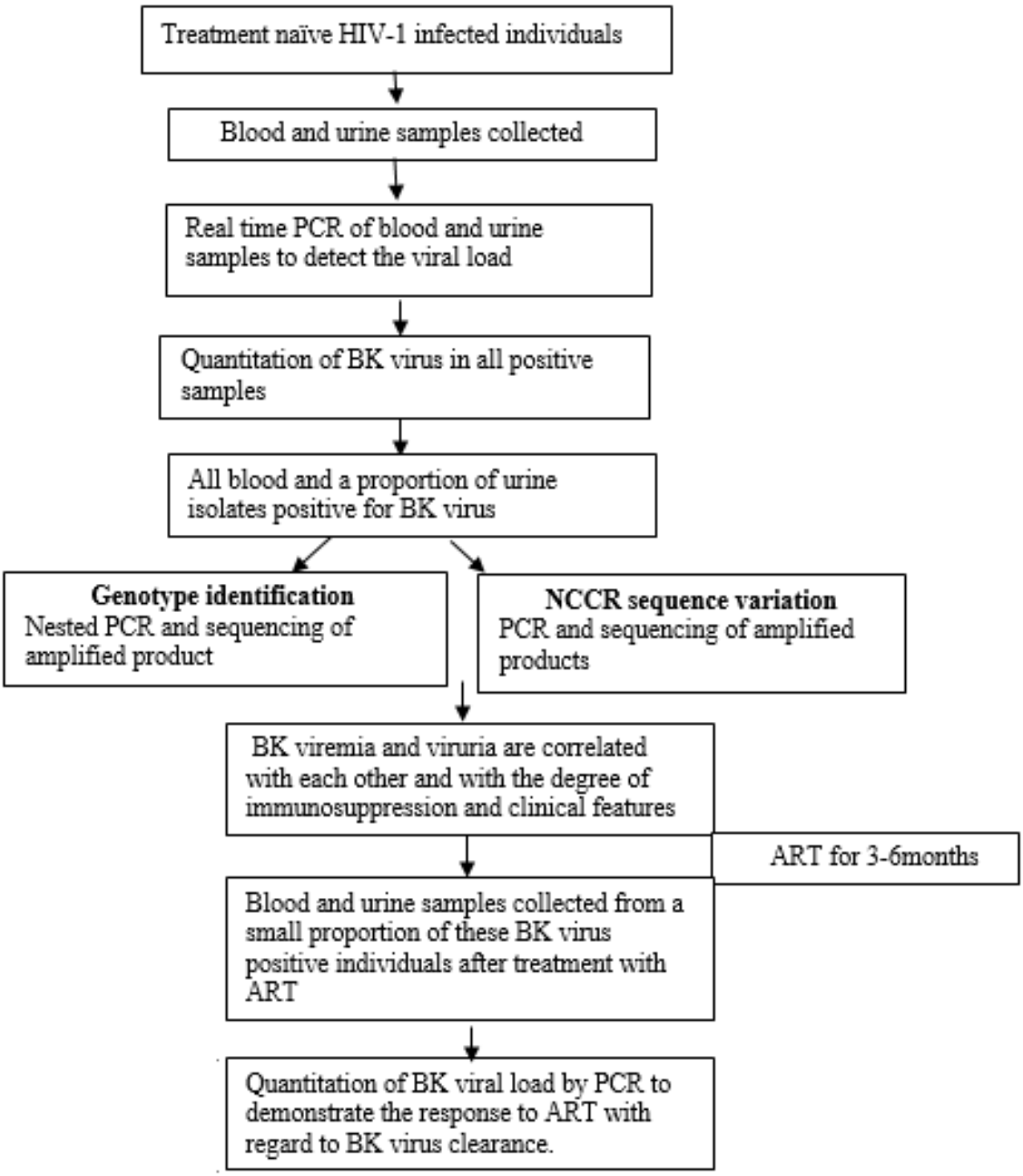
4.3 Clinical information of the study participants:

The detailed clinical information like, patient age, sex, regional identification, HIV related opportunistic infections (oral candidiasis, pulmonary tuberculosis, oral hairy leukoplakia, Cryptococcal meningitis, Disseminated tuberculosis, lymphoma, *Pneumocystis jirovecii (carinii)* pneumonia, CMV retinitis, Oesophageal candidiasis, wasting syndrome, Isosporiasis, etc) were collected from the individuals' hospital chart and they were classified in to different WHO clinical stages.

4.4 Samples tested:

1. Paired whole blood and Urine samples from treatment naïve HIV-1 infected individuals (n = 187)
2. Paired whole blood and Urine samples from HIV-1 infected individuals after a minimum of 3-6 months of ART (n=8)
3. Paired whole blood and Urine samples from normal healthy individuals (n = 93) (1 control for 2 patients)

Study algorithm



4.4.1 Whole blood samples:

A total of 6-8 ml of whole blood was collected in a sterile K2 EDTA BD vacutainers (New Jersey, USA) for the laboratory investigation from HIV-1 infected and healthy individuals. Fresh whole blood was used for CD4 estimation. In parallel whole blood was stored in multiple aliquots of 400µl and plasma also separated as required and made into multiple aliquots and stored at -70˚C until testing.

4.4.2 Urine samples:

10 to 20ml of urine was collected in a wide mouth screw capped container from Tarsons, Kolkata, India. After transferring 10 ml of urine into a sterile centrifuge tube, it was centrifuged in a refrigerated centrifuge at 4500 rpm for 10 minutes. The supernatant was discarded leaving 1ml with the deposit. After mixing the left over supernatant and deposit, it was stored in 2 micro centrifuge tubes with 200µl each and 1ml separately in another microcentrifuge tube. All the 3 tubes were stored at -70°C

4.5 Methods

4.5.1 CD4 estimation:

CD4 + T cell counts for the study samples were performed using the standard procedures by BD FACS Count system (Becton, Dickinson, San Jose, USA) with FACS Count CD4/CD4 SW, Version 1.0. The BD FACS Count system, using the BD FACS Count CD4/CD3 reagents, is a single test ready-to-use reagent tube pair containing the CD3monoclonal antibody with phycoerythrin (PE)-Cy5 (PE-Cy5) / CD4 with

phycoerythrin (PE) to determine the absolute number of helper/inducer T lymphocytes. The assay uses reference beads BD control kit low, medium, and high into which whole blood samples was added and the reading values to validate the system linearity.

When the whole blood is added to the reagents, fluorochrome- labeled antibodies containing the (CD3phycoerythrin (PE)-Cy5 (PE-Cy5) / CD4 phycoerythrin (PE)) in the reagents bind specifically to lymphocytes surface antigens. After a fixative solution is added to the reagent tubes, the sample is run on the instrument here the cells come in contact with the laser light, which causes the fluorochrome labeled cells to fluoresce. This fluorescent light intensity provides the information necessary for the instrument to count the cells. In addition to containing the antibody reagent, the reagent tubes also contain a known number of fluorochrome integrated reference beads. These beads function as a fluorescence standard for locating the lymphocytes and also as quantitation standards for enumerating the cells.

The 50µl of patient whole blood sample was added into the each reagent tubes containing the anti-human CD4 and anti-human CD3 monoclonal antibody reagents. Incubate the tubes for 60-120 minutes at room temperature (20 to 25°C) in dark condition. After the incubation add 50µl of fixative solution containing the 1% formaldehyde into each tube and again incubate for 30minutesat room temperature (20 to 25°C) in dark condition. The 50µl of low, medium, high control was added to the reagent tube before running the tubes on the instrument. If the control results are valid read the patient samples in the BD FACS count machine and results are generated as print outs.

4.5.1.1 Quality control for FACS count:

In order to avoid any diurnal variation all the blood samples tested for CD4 estimation are collected only on the day of testing between 8:00 -11:00 AM. In our laboratory, two internal quality control (IQC) samples were run every day. The previous day's samples with the lowest and the highest CD4 T cell counts were included as the controls. The samples were stored at room temperature (between 20° and 28°C). The IQC values of a given day were compared with the previous day's values and percent variation values were calculated. Percent variation was calculated as follows: (count on the first day/count on the second day) -1 ×100. A sample showing more than 20% variation from the previous day's value was considered not acceptable and the clinical samples were retested if necessary. Our daily quality control data from CD3 and CD4 T cell determinations show very good performance. In addition to 2 internal quality controls we also use commercial stabilized blood (BD Multi-Check control, Becton, Dickinson, San Jose, CA) as external quality control for routine quality control testing. The data were also analyzed for every run and the results were satisfactory (98).

Our laboratory is also a participating External Quality Assessment Scheme (EQAS) programs under NARI/NACO. Under this EQAS program, every year 2 batches of QC samples sent by National AIDS Research Institute, Pune, India (NARI)/NACO are estimated for their CD3, CD4 values using the Guava® Easy CD4™ System and BD FACSCount system the results obtained using our system was sent to NARI for evaluation. Since participation we always obtained values in acceptable range and passed the QC every time.

4.5.2 HIV-1 viral load estimation using Artus assay:

4.5.2.1 RNA extraction

The HIV-1 viral load estimation was done for the BKV positive samples using Artus RealArt™ HIV-1 assays (Qiagen Hamburg, Germany) using Rotor- Gene™ 6000 The QIAamp® Viral RNA kit was used for viral RNA extraction. The spin protocol was followed and before starting the assay following reagents was reconstituted with appropriate reagents.

- The carrier RNA must be added to the buffer AVL before use. (Conc. of carrier RNA is 10µg/ml lysis buffer) Carrier RNA dissolved in buffer AVL is stable for 6 months at 2-8ºC and no more than 2 weeks at room temperature (To avoid these make multiple aliquots). The carrier RNA added to the buffer AVL improves binding of viral RNA to the QIAamp membrane.
- Buffers AW1 and AW2 are supplied as a concentrate and appropriate amount of ethanol (96-100%) should be added before using them. They are stable for 1 year when stored at room temperature.
- The 560 µL of buffer AVL was added into a sterile 1.7 ml micro centrifuge tube and 140 µL of plasma was added to the buffer AVL in the microcentrifuge tube. Mixed by pulse-vortexing for 15 seconds and spun down at 8000rpm for 1 minute and then incubated at room temperature (15°C-25°C) for 10 minutes.

- The sample was first lysed using the lysis solution (Buffer AVL) under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA.
- Then 560µL of ethanol (96-100%) was added to the sample tubes. Mixed by pulse-vortexing for 15 seconds and spun down at 9000 rpm for 30 seconds. Buffering conditions are then adjusted to provide optimum binding of the RNA to the QIA amp membrane, which is a silica-gel-based membrane.
- 630µL of sample was transferred onto a QIA amp spin column without wetting the rim close the cap and centrifuged at 8,000 rpm for 1 minute. During this step the RNA binds to the silica-gel-based membrane.
- The QIAamp spin column placed into another sterile 1.5-ml collection tube and discarded the tube containing the filtrate.
- Again transferred the remaining 630µL to the QIA amp spin column and Spun at 8000 rpm for 1 minute then placed the QIA amp spin column into another sterile 1.5-ml collection tube and discarded the tube containing the filtrate.
- Then 500 µL of AW1 (wash buffer 1) was added and spun at 8000 rpm for 1 minute. Placed the QIAamp spin column into another sterile collection tube and discarded the tube containing the filtrate.
- Again 500 µL of AW2 (wash buffer 2) was added and spun at 14000 rpm for 3 minutes. Placed the QIAamp spin column into another sterile collection tube and discarded the tube containing the filtrate.

- During these steps the contaminants are washed away in two steps using two different wash buffers (AW1 and AW2). Placed the QIAamp spin column into a clean 1.5-ml collection tube (not provided) and discarded the tube containing the filtrate and spin at 14000 rpm for 2 minutes.
- The QIAamp spin column was placed into a sterile 1.5-ml micro centrifuge tube and added 50μL of elution buffer (RNase-free buffer (AVE)) that has been equilibrated to room temperature. Incubated at room temperature for 1 minute and then centrifuged at 8,000rpm for 3 minutes.
- The RNA is finally eluted out in a special RNase-free buffer (AVE). AVE has RNase-free water that contains 0.04% sodium azide to prevent microbial growth and subsequent contamination with RNases. Viral RNA is stable for up to one year when stored at –20°C or –70°C.

4.5.2.2 Master Mix reagent preparation for amplification:

Table 7: Reagents of Artus HI Virus-1 RG RT PCR kit

Reagent	Use
HIV -1 RG master A & B	Master mix
HIV -1 RG QS 1, 2, 3 & 4	PCR standards
HIV -1 RG IC	Internal control
Water	PCR grade

The amplification of HIV-1 was done using the commercial available reagent Artus HI Virus-1 RG RT PCR kit which includes the following reagents as shown in **Table 7**

- The cooling block must be pre-cooled to +4ºC before use.
- The desired number of flat top PCR tubes were placed in the cooling block.

- For 1 reaction add 12 µL of HIV-1 RG master A, 18 µL of HIV-1 RG master B and add 2 µL of HIV-1 RG IC (Internal control) were added to the sterile 1.7 ml micro centrifuge tubes.
- Calculated according to the number of samples and added appropriate reagents into the sterile 1.7 ml micro centrifuge tubes and added 30 µL of these master mix reagents into desired individual number of flat top PCR tubes.
- Then 20 µL extracted RNA was added into the appropriate labeled flat top PCR tubes containing the master mix reagents.

- These tubes were then placed into the Rotor gene RG-3000 machine(Corbett Research, Mortlake, Victoria, Australia)and start the assay using the Rotor gene software

The cycling conditions used for amplification are as shown **Table 8**

Table 8: Cycling conditions used for amplification in HIV load estimation by Artus assay

Feature	Temperature (ºC)	Time (seconds)
Hold 1	50	1800
Hold 2	95	900
Cycling (50 cycles)	95	30
	50	60
	72	30

- Hold 1, hold 2 and cycling was set to high speed when adjusting the profile (except the hold at 50°C for 60 seconds which is set at normal speed)
- Sample numbers should then be entered and after completion of the run, the finding of "threshold" to determine the Ct value for the standards and each sample was done.
- Any sample that was negative, checked for possible PCR inhibition. This was done by checking for any signals in the Cycling A ROX channel.
- Absence of signal in both the channels indicates PCR inhibition and the sample must be repeated.

Quality control: Apart from the standards provided by the kit a characterized low copy HIV-1 (10^3 copies/ml) sample was used as an in-house positive control. In all the runs the in-house control gave satisfactory result. Our laboratory is also a participating External

Quality Assessment Scheme (EQAS) programs under NARI/NACO and with UKNEQAS. The last years' performance was satisfactory.

4.6 BK specific methods:

4.6.1 DNA Extraction:

DNA was extracted from whole blood and urine samples using the commercially available QIA amp DNA blood mini kit (Qiagen, Hilden, Germany). The manufacturer's instructions were strictly followed for the extraction of DNA.

The spin protocol was followed for DNA extraction from whole blood and urine samples. Before starting extraction the following reagents reconstituted with appropriate reagents.

- Protease reconstituted using the 5.5 ml protease solvent into the lyophilized vial containing the Qiagen Protease. Reconstituted Qiagen protease is stable for up to 2 months when stored at 2–8°C.
- Buffers AW1 and AW2 are supplied as a concentrate and appropriate amount of ethanol (96-100%) were added before using them. They were stable for 1 year when stored at room temperature.

<u>Steps of DNA extraction:</u>

- The 20 µl of protease was added into the sterile 1.7 ml micro centrifuge tube and 200 µl of sample was added.
- Then 200 µl of buffer AL was added to the sample, mixed by pulse-vortexing for 15 seconds and incubated in dry bath at 56°C for 10 minutes.

- The micro centrifuge tube was centrifuged to remove drops from the inside of the lid and added 200 µl of ethanol (96–100%) then mixed again by pulse-vortexing for 15 seconds.
- The sterile 1.7 ml micro centrifuge was again centrifuged to remove drops from the inside of the lid and carefully transferred the mixture to the QIA amp Mini spin column without wetting the rim.
- The QIA amp mini spin column was centrifuged at 8000 rpm for 1 minute and placed the QIA amp mini spin column to a sterile 2 ml collection tube and discarded the tube containing the filtrate.
- The 500 µL of AW1 (wash buffer 1) was added and spin at 8000 rpm for 1 minute. The QIAamp spin column was placed into another sterile 1.7-ml collection tube and discarded the tube containing the filtrate.
- Again the 500 µL of AW2 (wash buffer 2) was added and spin at 14000 rpm for 3 minutes. The QIA amp spin column was placed into another sterile collection tube and discarded the tube containing the filtrate.
- During this step the cellular debris are washed away in two steps using two different wash buffers (AW1 and AW2). The QIA amp spin column was placed into a clean collection tube and discarded the tube containing the filtrate and spin at 14000 rpm for 1 minute.
- Finally the QIA amp spin column was placed into a sterile 1.7 ml micro centrifuge tube and added 200µL of elution buffer (AE) that has been equilibrated to room

temperature. The tube was incubated at room temperature for 1 minute and then centrifuged at 8,000 rpm for 1 minute.

- The DNA eluted at the end of the extraction procedure was stored in aliquots at -30˚C freezer until testing.

4.6.2 In-house real time Real time PCR for BK virus (BKV):

A qualitative real time PCR assay carried out for BKV in the study samples. The primer and probe sequences and the gene targeted used for the study is shown with references

Table 9: Primers and probe sequences specific for VP3 gene of BK virus (99)

Primers	**Sequences**
Forward	5 '-TGTACGGGACTGTAACACCTGC-3'
Reverse	5'-TTTGGMACTTGCACGGG-3'
Probe	5'-ROX-TGAAGCATTGAAGATGGCCCCAAC-BHQ2-3'

The real time PCR was initially standardized with ROX dye as the fluorophore for BKV probe. The real time PCR used in this study was based on the Taq man chemistry. The Taq man probes are the dual labeled probes with short oligomers tagged with florescent reporting dye attached at 5' end and a quencher molecule attached at the 3' end. Since the

probe sequences are only 15-20 base pair length the reporter and quencher dye are in close proximity with each other resulting in little or no detectable florescence. During the amplification cycling process, the Taq DNA polymerase enzyme extends from each primer and due to its exonuclease activity which cleaves the downstream Taq man probe resulting in separation of reported dye from quencher. During every cycle the reporting dye gets separated with detectable fluorescence which is proportional to the amount of accumulated PCR product.

BKV plasmid was used as the sources of positive controls for BKV. Sterile Milli-Q water control was included after every 5th place to check the PCR cross contamination. Real-time PCR was carried out [Rotor gene RG-3000 or RG-6000 (Corbett Research, Mortlake, Victoria, Australia)]. The reaction mix and cycling conditions used for the real PCR of BK virus is represented in **Tables 10 and 11** respectively.

Table 10: Reaction mix for in house real time PCR of BK virus

Reaction mix	**Volume**
QuantiTect Multiplex PCR NoROX master mix	12.5 µl
BKV Forward primer (7.5 picomoles)	0.075 µl
BKV Reverse primer (7.5 picomoles)	0.075 µl
Taqman probe (5 picomoles)	0.05 µl
Extracted DNA	10 µl
Total	**15 µl**

Table 11: Cycling conditions for in house real time PCR of BK virus

Feature	Temperature	Time (seconds)
Hold	95ºC	900
Cycling (50 cycles)	95ºC	45
	60ºC	75

4.6.2.1 In house real time Quantitation PCR for BK virus:

Any sample positive in the screening qualitative multiplex real-time PCR was tested by in-house real time quantitation PCR. The PCR standards were prepared using plasmids of respective targets cloned using Topo TA Clone/TOP10 kit, (Invitrogen, Carisbad, USA). The plasmids were quantitated using the DNA spectrophotometry (BioTek Instruments, Vermont, USA) and then diluted serially in tenfold dilutions using milliQ water to obtain 10^6 to 10^2 copies/ µl. The plasmids were aliquoted in multiple tubes and stored at -30°C freezer. Quantification of viral DNA was carried out using the same protocol as qualitative PCR with respective primers and probes. The thermal cycling conditions used were same as those for qualitative PCR

The limit of detection for BK virus using In house real time PCR was ≥1 plasmid/10µl input.The BKV primers and probes did not show any cross reactivity with other DNA viruses like EBV, CMV, HSV-1, HHV-6, VZV, JC, HSV-2, HHV-8 and Adenovirus (100,101)

4.6.3 Real time PCR for Endogenous Retrovirus-3 (ERV-3):

An in-house ERV-3 (endogenous retrovirus) qualitative real time PCR was carried out to check the DNA integrity for all PCR negative samples. The primers and probe was taken from the previously published literature (102).

Table 12: ERV-3 specific primer and probe sequences for real time PCR

Primers	**Sequences**
Forward	PHP10-F: 5'- CATGGGAAGCAAGGGAACTAATG
Reverse	PHP10-R: 5'- CCCAGCGAGCAATACAGAATTT
Probe	PHP-P505: 5'- TCTTCCCTCGAACCTGCACCATCAAGTCA

Table 13: Reaction mix ERV-3 real time PCR

Ingredients	**For 1 reaction**
Multiplex NOROX mix	12.5µl
ERV-3 forward (100pm/µl)	0.075µl
ERV-3 reverse (100pm/µl)	0.075µl
ERV-3 probe (100pm/µl)	0.05µl
Water	2.3µl
Total	**15µl**

This assay was also based on the Taqman chemistry carried out using the reaction mix as mentioned in **Table 13** with ERV-3Taqman probes containing FAM dye as its fluorophore.

The thermal cycling conditions used were for this assay was 95°C for 15 min, 95°C for 45 sec, and 60°C for 75 sec for 50 cycles. The assay was performed using the Rotor gene RG-6000 (Corbett Research, Mortlake, Victoria, Australia)

4.7 Genotyping and Analysis of BKV NCCR rearrangement methods

The same extracted DNA utilized for real time PCR was used for genotyping and analysis of BKV NCCR rearrangement from a proportion of BKV positive samples to determine the prevalent genotypes and to look for NCCR rearrangement seen among HIV-1 infected individuals.

4.7.1 Amplification of NCCR region:

The amplification was carried out in a nested PCR format. The primer sequences were taken from published literature (53)

Table 14: Outer and inner primers of BKV NCCR

Outer primers	BKTT5 5'-GAGCTCCATGGATTCTTC-3'
1st round	BKTT6 5'-CCAGTCCAGGTTTTACCA-3'
Inner primers	BKTT7 5'-CCCTGTTAAGAACTTTATCCATTT-3'
2nd round	BKTT8 5'-AACTTTCACTGAAGCTTGTCGT-3'

The PCR was carried out in thermal cyclers, PTC-100 (MJ research, California, USA)/Veriti (Applied Biosystem, California, USA). During the first round of PCR the following reaction mix shown in **Table 15** was used.

Table 15: Reaction mix for the 1st round of NCCR amplification

Ingredients	**For 1 reaction (µl)**
Hot star taq master mix	25
Outer forward	0.25
Outer reverse	0.25
Water	19.5
Extracted DNA	5
Total	**50 µl**

Table 16: Cycling conditions used for amplification of NCCR by nested PCR

Feature	**Temperature**	**Time**
Enzyme activation	95°	15 minutes
Denaturation (40 cycles)	94°C	1 minute
Annealing (40 cycles)	55°C	1 minute
Extension (40 cycles)	72°C	1 minute
Final extension	72°C	10 minutes

The following cycling conditions were followed: 95°C for 15 min followed by 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 40 cycles with final extension cycle at 72°C for 10 min as given in **Table 16.**

For the 2nd round of amplification 5 µL of 1st round PCR product was added to the sterile PCR tubes containing the 2.5 units of Hot star taq master mix (Qiagen, Hilden, Germany) and 25 picomoles of respective inner forward and inner reverse primers in a total reaction volume of 50 µL. The cycling conditions used are same as followed for first round of amplification.

4.7.2 Agarose gel electrophoresis:

The amplified PCR product was detected using agarose gel electrophoresis. A 2% agarose (Sigma Aldrich Inc, St Louis, MO, USA) gel was prepared using 100 ml of 1X TAE (Tris acetate EDTA buffer) containing 0.5 µg/mL of ethidium bromide. The prepared 2% agarose gel was poured over the gel casting tray with appropriate gel comb. After 30-40 minutes the gel was placed into the electrophoresis tank equipment (Cleaver Scientific ltd, Warwickshire, England) containing the 1X TAE (Tris acetate EDTA) buffer and removed the gel comb. The 5 µL of PCR product was mixed with 3 µL of tracking dye (bromo phenol blue with sucrose) and added to appropriate wells. The gel was loaded with 100-1200 base pair range molecular weight marker (Bio basic inc, Ontario, Canada) to identify the PCR base pairs and run the gel placed in the electrophoresis equipment (Cleaver Scientific ltd, Warwickshire, England) at 120 Volts for 40-50 minutes. The PCR product was detected using the gel documentation system (Gel Doc, Bio Rad, California,USA) and

the base pairs was analyzed using the Quantity One software version 4.1.1 (BioRad, California, USA).

4.7.3 Pre-cycle sequencing clean-up:

- Pre-cycle sequencing clean-up was done using the Millipore sequencing clean up assay (Montage, MA, USA).
- The Millipore plates with required number of wells were labeled for Pre-cycle sequencing clean up.
- The second round PCR amplified product was made up to 100 µl final volume by adding sterile milli Q water.
- The contents were mixed well and transferred onto the appropriately labeled well in the pre-sequencing Millipore plate.
- The Millipore plate was connected to vacuum pump and applied negative pressure till the well is completely dry.
- 100 µl of sterile milli Q water was added and the previous step was repeated.
- 20 µl of the sterile milli Q water was added and the plate was placed on the shaker (Thermo Scientific, Barnstead/lab-line, Ashville, USA) for 2 minutes.
- The plate was mixed and the entire contents were aspirated into a sterile 0.2ml PCR tube.

4.7.4 Sequencing PCR reaction:

- The primers used for sequencing PCR were made up to1 picomole/µl concentration using sterile nuclease free water.
- Forward and reverse primer reactions of respective viruses are set separately for sequencing PCR.
- The sequencing PCR was carried out using the Big Dye Terminator Cycle Sequencing assay kit (Applied Biosystems, Foster City, CA).
- The sequencing reactions were prepared with following reagents as shown below

Table 17: Reagents used for NCCR sequencing PCR

Reagents	**For 1 reaction**
Pre-sequencing clean-up product	1.0 µl
Primer (1picomole/µl)	3.2 µl (each primer)
RR Master Mix	1.0 µl
RR Buffer	2 µl
Pyrogen free water	2.8 µl
Total	**10 µl**

Aliquoted 9 µl of the prepared master mix into fresh 0.2ml PCR tubes and 1 µl of the pre sequenced product was added to make up to a final volume of 10 µl. The sequencing PCR

was carried out in thermal cyclers , PTC-100 (MJ research, California, USA) or Veriti (Applied biosystem, California,USA), with the following cycling conditions: 96 ºC for 15 seconds, 50ºC for 20 seconds, 60 ºC for 4 minutes for 25 cycles and extension cycle at 15 ºC for 30 minutes.

4.7.5 Post-cycle sequencing clean-up:

- The Millipore plate was labeled with required number of wells for Post-cycle sequencing clean up.
- The 30 µl of Millipore injection solution was added to sequence amplified product to make up to 40 µl and transferred to appropriately labeled post-sequencing Millipore plate.
- The Millipore plate was connected to vacuum pump and applied negative pressure till the well is completely dry.
- The 40 µl of the Millipore injection solution was added and the previous step was repeated.
- The 30 µl of injection solution was added and mixed well using the titer plate shaker (Thermo Scientific, Barnstead/lab-line, Ashville, USA)
- Aspirated the entire contents into a sterile 0.6ml tube and loaded the tubes into the ABI 310 genetic analyser (Applied Bio systems, Foster City, CA).

The ABI 310 Genetic Analyzer (Applied Bio systems, Foster City, CA) contains a capillary cathode and anode. The capillary tube was designed to touch the sample at the cathode

end while the anode end kept in the EDTA buffer. During the electrokinetic injection current is passed from the cathode to anode. The sample passes through the detector window and the laser gets excited when the individual labeled fluorescent dyes passes at that point the fluorescence signal reading was taken. The software installed in the computer converts this signal in the form of electropherogram. The sequence in the form of electropherogram for respective viruses was bidirectionally aligned using the Finch TV software (version 1.4.0) and Bio Edit sequence alignment editor software version 7.0.9.0. These bi directionally aligned sequences are utilized for phylogenetic analyses using neighbour joining method with 1000 bootstrap replicates in MEGA4 software (version 4.0).

4.8 Control samples

Blood and urine samples were collected from 93 (1 control for 2 patients) age and sex matched (as of patients) healthy individuals taken as controls, after obtaining informed consent for these individuals. The samples were processed in the same way as the patient samples and stored at -70°C.

A separate informed consent was obtained from these individuals for HIV testing, which was done on their plasma samples using ErbaSure HIV gen4, a fourth generation in vitro enzyme immunoassay.

4.8.1 HIV testing on control samples

HIV testing on all control samples was performed using ErbaSure HIV gen4 ELISA, a fourth generation ELISA. It was done according to the manufacturer's instructions.

1. The required amount of strips were taken in the frame. (microtitre plate)

2. The negative controls (100µl) were added in wells 1A to 1D, antibody positive control (100µl) into well 1E and antigen positive control (100µl) into well 1F respectively.

3. 100µl of the samples to be tested were added into the remaining wells

4. The plate was incubated at 37°C±1°C for 60 minutes after sealing the plate with a sealer

5. Before the last 10 minutes of incubation, a 1:51 dilution of conjugate with conjugate diluents is made

6. After aspirating the contents from the wells, each well was washed 5 times with diluted washing solution (350 µl/well/time)

7. The plate was tapped on the adsorbent paper to remove excess washing solution and 100µl of prepared diluted conjugate was added into each well.

8. The plate was incubated at 37°C±1°C for 30 minutes after sealing the plate with a sealer

9. Before the last 5-10 minutes of second incubation, a 1:101 dilution of substrate with substrate buffer is made

10. After aspirating the contents from the wells, each well was washed 5 times with dilutrd washing solution (350µl/well/time)

11. The plate was tapped on the adsorbent paper to remove excess washing solution and 100µl of prepared substrate is added into each well and incubate at room temperature for 30 minutes avoiding exposure to light

12. 100 µl of stop solution was added into each well and the plate is tapped gently to homogenize the coloring materials.

13. The absorbance was read at 450nm (reference wavelength at 620nm) against air within 30 minutes after pipetting of stop solution.

Interpretation of results:

1. The mean of the negative control (NC) is calculated

2. The cut off value is calculated as mean of NC + 0.2

3. Any absorbance value above the cut off value was considered reactive to anti- HIV or HIV antigen.

Only HIV negative control samples were taken for further testing. In house Qualitative BK PCR was done on these samples as done for patient samples. Quantitation was done on the positive samples and negative samples were subjected to quantitative ERV-3 PCR to check the DNA integrity.

4.9 Statistical analysis

Chi^2 test was used to see the difference in proportion of BKV positive status among 2 groups of HIV-1 infected individuals and HIV negative healthy controls. The two tailed t test was used to analyze the difference in BKV viral load among the 2 groups of HIV-1 infected individuals and controls. The two tailed t test probability, Welch test (for assuming unequal variance of independent sample) were used to analyze the viral load level and spearman's test was used to determine the correlation coefficient. The data generated from the study samples using these statistical methods were plotted using the Box-and-whisker plot. The percentile value, Chi^2 and the two tailed t test analysis were done using the Medcalc software version 9.2.0.1. (http://www.medcalc.be). Analysis of variance for one way ANOVA was done using Epi Info 6 version 6.04b. The univariate and multivariate analysis of all the clinical and laboratory data were performed using STATA 10.0 (Stata Corp, College Station, TX). A p value of < 0.05 was considered as significant.

5. Results

Urine and whole blood samples which were collected from 187 treatment naïve HIV-1 infected individuals and 93 HIV negative healthy individuals were tested during the study period.

Findings on samples collected from HIV-1 infected and healthy individuals:

5.1 Demographic details:

Among the total 187 treatment naïve HIV-1 infected individuals, 86 were females and 101 were males as represented in **Figure 9**. The patients' age group ranged from 22-68 years with the mean age being 40 years (mean age in females = 38yrs; males = 41yrs). The age distribution of HIV-1 infected individuals has been shown in **Table 18.**

These individuals were from different geographic regions, majority were from Tamilnadu (n=114, 60.9%) followed by Andhra Pradesh (n=46, 24.5%) and West Bengal (n=13, 6.7%). The rest of the patients were from other regions as shown in the **Table 19**

Out of 93 age and sex matched healthy individuals collected, (1 control for two patients) 43 were females and 50 males as represented in **Figure 10**. All these individuals were from the state of Tamilnadu.

Figure 9: The pie chart showing the sex distribution of study population (HIV-1 infected individuals)

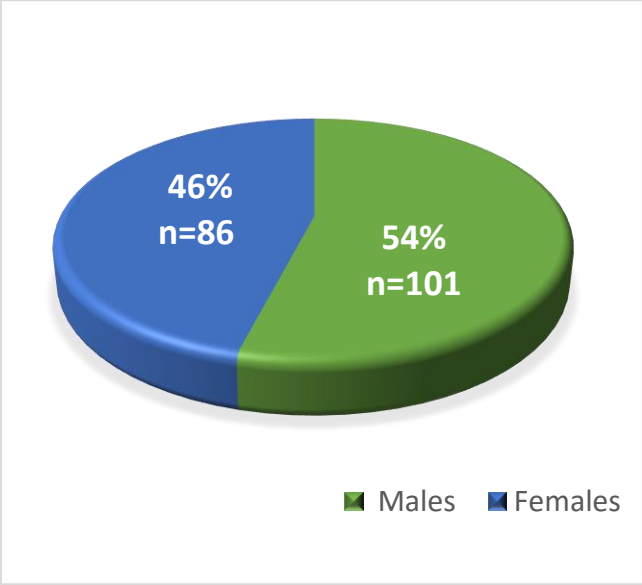


Table 18: Age distribution of study subjects (HIV-1 infected individuals)

Age group in years	Number of subjects (%)
20 – 30	26 (13.9%)
31 – 40	82 (43.8%)
41 -50	53 (28.3%)
51 – 60	20 (10.6%)
61 -70	6 (3.2%)
Total	**187 (100%)**

Table 19: Geographic distribution of study population

Region	Number	Percentage
Tamilnadu	114	60.9
Andhra Pradesh	46	24.5
West Bengal	13	7
Jharkhand	4	2.1
Bihar	3	1.6
Kerala	2	1
Chhattisgarh	1	0.5
Orissa	1	0.5
Tripura	1	0.5
Assam	1	0.5
Andaman and Nicobar islands	1	0.5
Total	187	100

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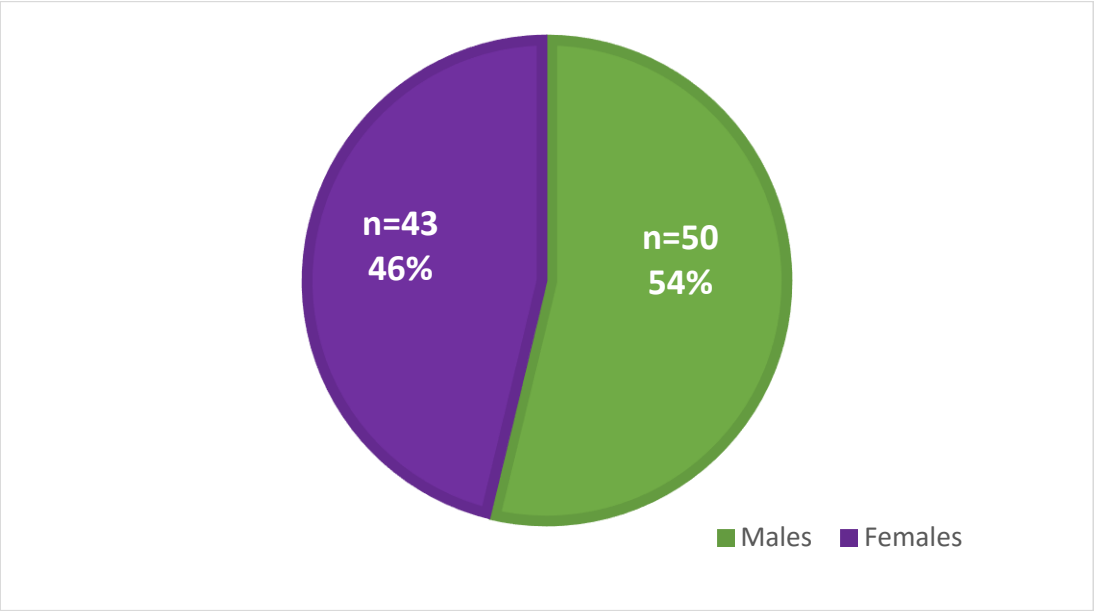
Figure 10: Pie chart representing the sex distribution of healthy controls.



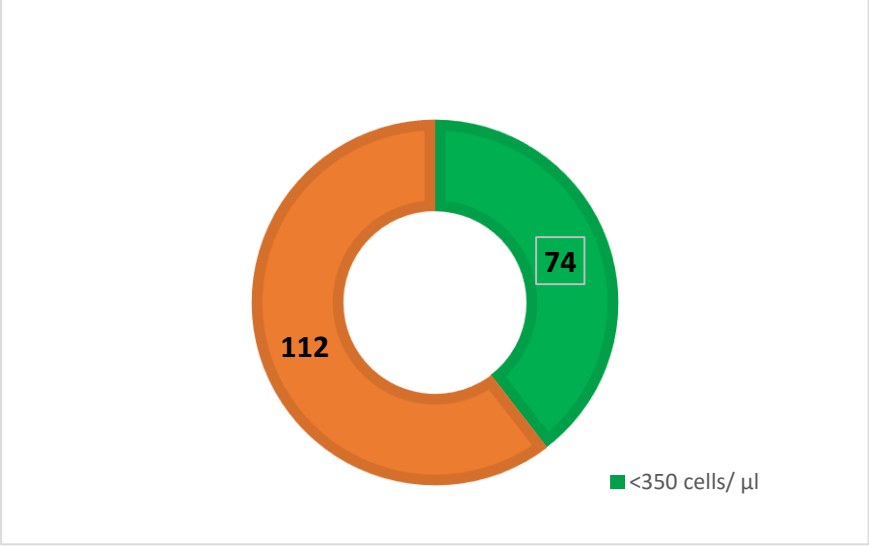
5.2 CD4 counts

The internal quality control values obtained in all the runs were satisfactory. The CD4 counts of 186 HIV-1 infected individuals were only available for analysis as there was a technical error for one of the sample and the CD4 value could not be obtained. The mean (Range 7 to 1300 cells/µl) the median CD4 count were 418 and 436 cells/ µl respectively. Of these 186 subjects, 74 had CD4 counts less than 350 cells/ µl and 112 above 350 cells/ µl. This was used as a criterion to collect follow up samples**.** The distribution of HIV-1 infected individuals according to CD4 counts with 350 cells/ µl as the cut off has been represented in **Figure 11**

5.2.1 CD4 counts of study participants based on CDC categories

According to CDC classification of CD4 counts, treatment naïve HIV-1 infected individuals were classified into 3 categories. 69 (37.1%) individuals had CD4 counts >500 cells/ µl and were classified under category 1 with the mean and the median CD4 count being 676 and 641 cells/ µl respectively. The CD4 count in this group range from 501-1300 cells/ µl. A total of 74 (39.8%) individuals were under category 2 (200-499 cells/ µl) who had CD4 counts ranging from 204-498 cells/ µl. The mean and the median CD4 count in this group is 373 and 376 cells/ µl respectively. Forty three (23.1%) out of 186 individuals were classified under category 3 (<200 cells/ µl) with mean and median CD4 count being 81 and 64 cells/ µl respectively.

Figure 11: Distribution of study subjects (treatment naïve HIV-1 infected individuals) based on CD4 counts taken for initiation of treatment (350 cells/ µl)



5.2.2 Distribution of study subjects (HIV1 infected individuals) based on CDC classification of HIV

Based on the CDC classification of HIV, of the total 69 under CD4 category 1, 56 (81.2%) individuals were under clinical category A, 12 (17.3%) under category B and one (1.4%) individual under category C. In CD4 category 2, among the total 74 individuals, 53 (71.6%) were under clinical category A, 16 (21.6%) individuals under clinical category B and five (6.8%) individuals under clinical category C. Of the total 43 subjects in CD4 category 3, six (7%) were under clinical category A, 16 (37.2%) under category B and 21 (48.8%) individuals under clinical category C.

The mean and the median values of study subjects'CD4 counts in each CDC category has been given in **Table 20.** The distribution of HIV-1 infected individuals based on CDC classification of HIV is shown in **Table 21**

The distribution of treatment naïve HIV-1 infected individuals based on CDC categorization of CD4 counts has been shown in **Figure 12.**

Figure 12: Distribution of treatment naïve HIV-1 infected individuals based on CDC categorization of CD4 counts in study subjects

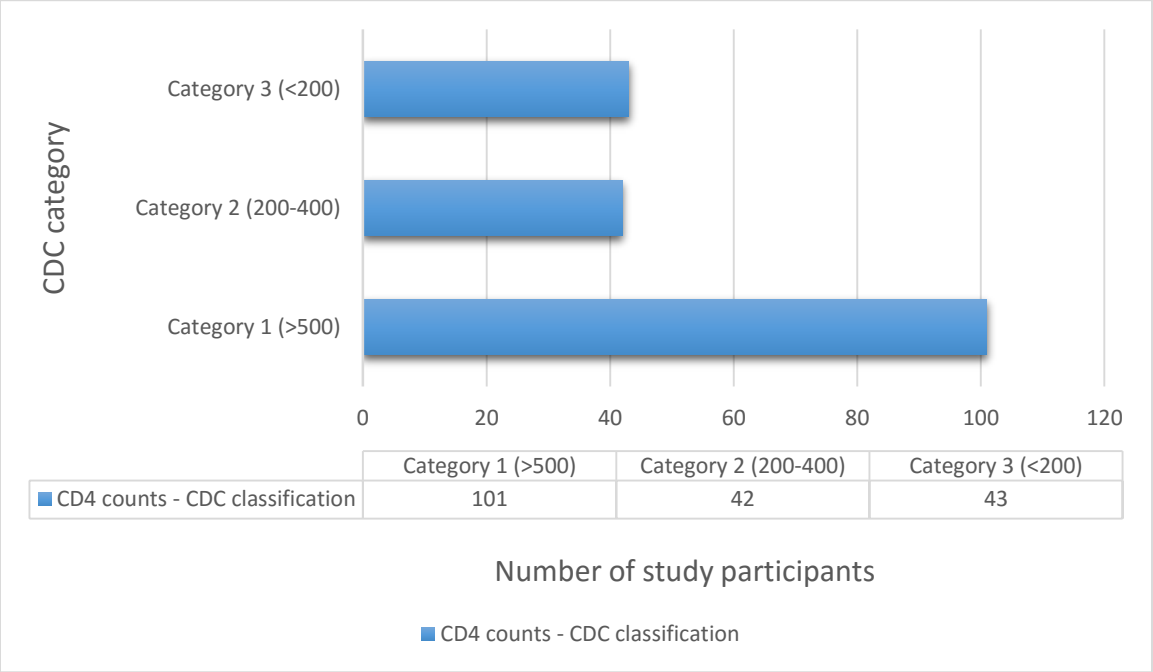


Table 20: Mean and Median of CD4 counts of HIV-1 infected individuals in each CDC category

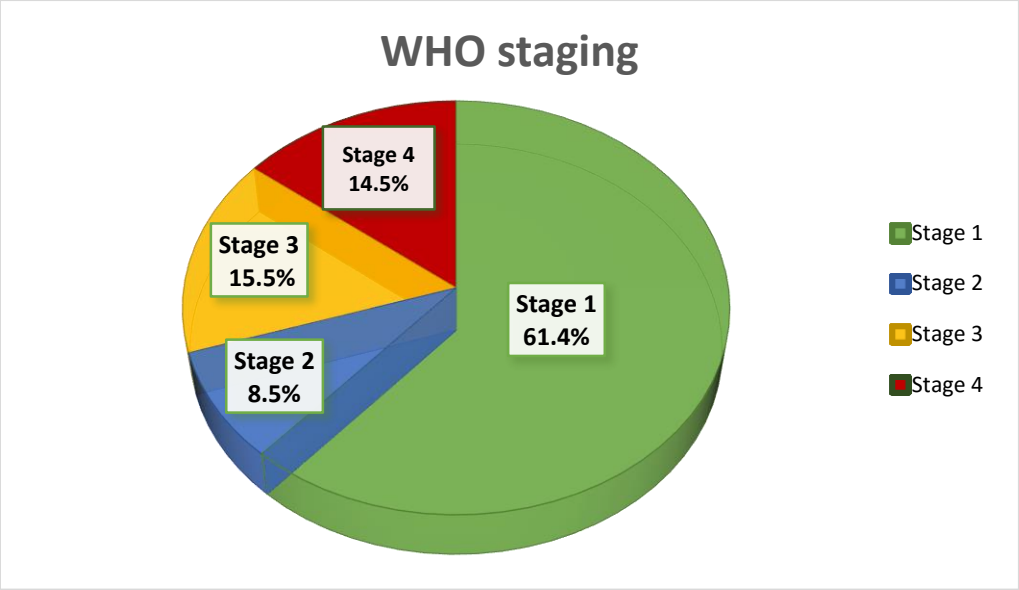
CDC category	CD4 counts of study subjects in cells / µl	
	Mean	Median
(1) > 500 cells/ µl	676	641
(2) 200-499 cells/ µl	373	376
(3) <200 cells/ µl	81	64

Table 21: Distribution of study subjects (HIV-1 infected individuals) based on CDC classification of HIV

CD4 count categories	Clinical categories		
	Category A, n (%)	Category B, n (%)	Category C, n (%)
(1)> 500 cells/ µl	A1, n=56 (30.1%)	B1, n=12 (6.4%)	C1, n=1 (0.53%)
(2)200-499 cells/ µl	A2, n=53 (28.5%)	B2, n=16 (8.6%)	C2, n=5 (2.6%)
(3)<200 cells/ µl	A3, n=6 (3.2%)	B3, n=16 (8.6%)	C3, n=21 (11.2%)

5.3 WHO clinical staging of HIV-1 infected individuals

Figure 13: Distribution of treatment naive HIV-1 infected individuals based on WHO clinical staging.



The clinical findings of HIV-1 infected individuals were collected from their hospital charts and these individuals were classified into 4 WHO clinical stages (1-4)

Out of the 187 study participants, 115 were classified into stage 1 of which 113 were asymptomatic and the remaining 2 subjects had persistent generalized lymphadenopathy. The CD4 counts in this group ranged 92-1300 cells/µl. A total of 16 individuals were classified into WHO clinical stage 2. They had varied clinical manifestations such as Herpes zoster and oral ulcerations with CD4 counts ranging from 43-636 cells/ µl.

Twenty nine individuals were categorized as stage 3, who had CD4 counts ranging from 28-769 cells/ µl. Majority of them presented with Oral candidiasis followed by Pulmonary tuberculosis, unexplained persistent fever, severe weight loss and unexplained thrombocytopenia. A total of 27 individuals were grouped under clinical stage 4, who presented with extra pulmonary tuberculosis, oesophageal candidiasis, disseminated tuberculosis, invasive cervical carcinoma and CMV retinitis. 25 out of 27 individuals had CD4 counts < 350 cells/ µl ranging from 7- 340 cells/ µl. The remaining two individuals had CD4 count of 455 and 529 cells/ µl respectively.

The distribution of study subjects (HIV-1 infected individuals) based on WHO clinical staging is represented in **Figure 13**

WHO staging and the respective clinical manifestations observed in HIV-1 infected study participants are represented in **Table 22**

Table 22: WHO staging and respective clinical manifestations observed among HIV-1 infected individuals

WHO stage (n)	Range of CD4 count (cells/ µl)	Clinical manifestations observed
1 (n=115)	92-1300	Asymptomatic (n=113) Persistent generalized lymphadenopathy (n=2)
2 (n=16)	43-636	Herpes zoster and oral ulcerations
3 (n=29)	28-769	Oral candidiasis, Pulmonary tuberculosis, unexplained persistent fever, severe weight loss and unexplained thrombocytopenia.
4 (n=27)	7-340	Extra pulmonary tuberculosis (Lymph node tuberculosis) Oesophageal candidiasis, disseminated tuberculosis, invasive cervical carcinoma and CMV retinitis

One way ANOVA analysis showed that there was a significant difference in mean CD4 count among the four WHO clinical stages of HIV. (*P* value < 0.001)

5.4. HIV-1 RNA estimation

The plasma HIV-1 viral load was determined for all BKV DNA positive samples (n=46) and a proportion of BKV negative samples (n=56). HIV-1viral load in BKV positive individuals ranged from 71 – 5888659 copies/ml with the mean viral load of 343405 copies/ml. The inter assay variation of the HIV-1 real time PCR assay was estimated and was found to be 13%.

The HIV RNA load and the corresponding CD4 range according to WHO clinical staging of HIV is represented in **Table 23**

Table 23: HIV-1 viral load and corresponding CD4 range according to the WHO clinical stages of HIV

WHO clinical stage (n)	**HIV-1 viral load range (copies/ml)**	**Mean log_{10} viral load ± SD**	**CD4 count range (cells/ µl)**
1 (n=66)	71-854028	3.66 ± 1.13	116-1300
2 (n=5)	108–100816	3.66 ± 1.13	206-584
3 (n=17)	71-489718	4.03 ± 1.32	57-769
4 (n=14)	358–5888659	5.0 ± 1.28	24-455

One way ANOVA analysis suggested there was a significant difference in mean of HIV-1 viral load among four WHO clinical stages of HIV. (P value <0.001*)*

5.5 BK virus specific results and analysis

5.5.1 Urine samples

Real time PCR for BK virus has already been standardized and been used for routine diagnostic use. The runs were validated based on External and internal quality controls. The inter assay variation of the BKV real time PCR assay was estimated and was found to be 7.5%. Out of 187 samples from HIV-1 infected individuals, 48 samples were positive for BKV DNA with qualitative real time PCR. Further when these 48 samples were subjected to in house quantitative real time PCR, 46 (25.6%) samples had BK viral load ranging from 1- 359886 copies/ ml with the mean log10 viral load of 14322 copies/ml. Two samples were negative for BKV DNA on quantitation even after repeat testing.

Out of the 93 healthy individuals who were HIV-1 negative, samples from10 (10.7%) individuals were positive for BKV DNA with viral load ranging from 1- 572 copies/ml. The mean viral load in this group was 107 copies/ml. The demographic details and BK viral load of healthy controls is given in **Table 24**

Comparison of BK virus positivity of urine in HIV-1 infected and healthy HIV negative individuals showed significant difference **(*P* value 0.003)**

5.5.2. Whole blood samples

Out of the 187 whole blood samples from HIV-1 infected individuals, only 2 (1%) samples were positive for BKV DNA with viral load of 1 copy/ml each.

BKV DNA positivity was not seen in any of the blood samples from healthy individuals. The percentage of positivity and their mean viral loads detected by real time PCR from the urine and whole blood samples in the HIV infected and healthy individuals is shown in **Table 25**

Table 24: Demographic details and BK viral load of HIV negative healthy controls

S.no	Study No	Age in Years / sex (M/F)	BK viral load (copies/ml)
1	HBKUC 26	34/M	2
2	HBKUC 46	47/F	7
3	HBKUC 50	39/F	2
4	HBKUC 51	58/M	34
5	HBKUC 56	43/M	9
6	HBKUC 63	29/M	240
7	HBKUC 77	35/F	1
8	HBKUC 91	43/F	572
9	HBKUC 92	39/F	12
10	HBKUC 93	28/F	192

Table 25: The number of BKV positive urine and whole blood samples and their mean viral load among HIV-1 infected and HIV negative healthy individuals.

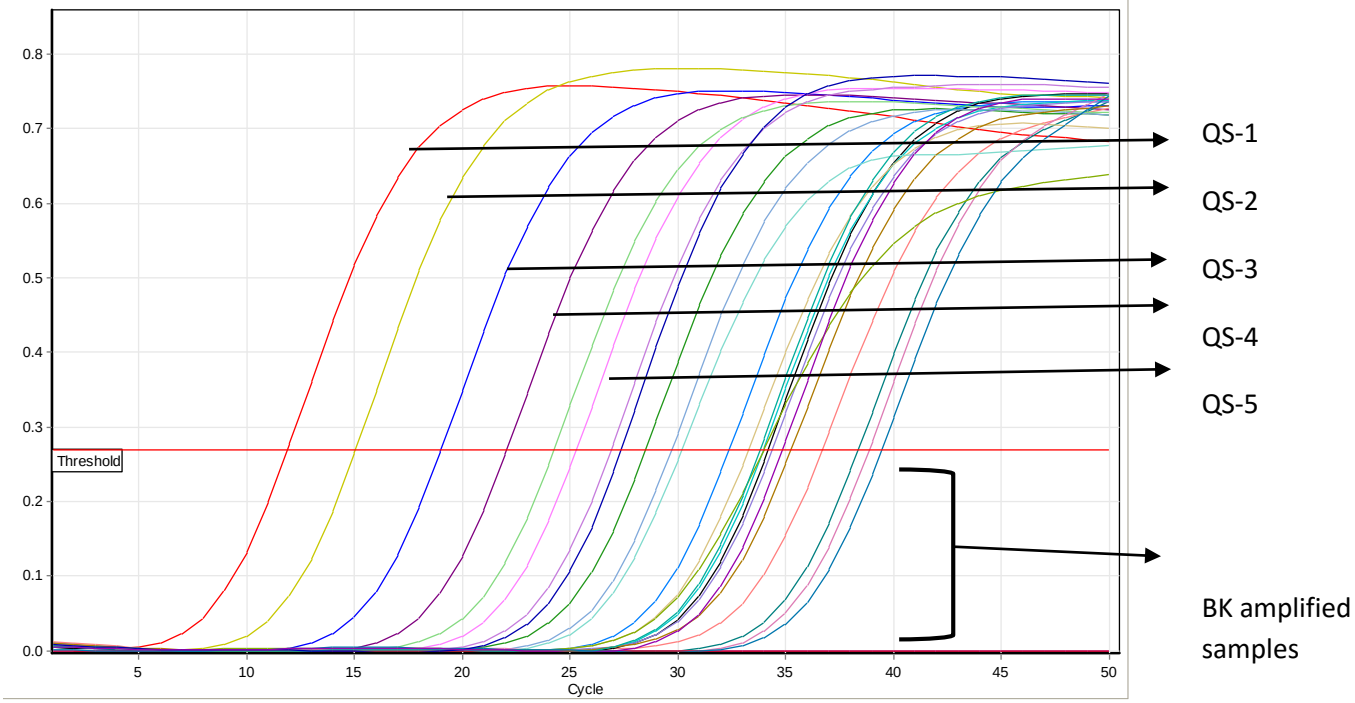
Study population	Samples	Total (n)	BKV DNA positive, n (%)	Viral load range (log_{10} copies)	Mean viral load Mean log_{10}±SD
HIV-1 infected	Urine	187	46 (25.6%) #	0 - 5.55	2.17±1.5
	Blood	187	2 (1%)	0	0
HIV negative healthy controls	Urine	93	10 (10.7%) #	0 – 2.75	1.24±0.96
	Blood	93	0	0	0

#Comparison of BK virus positivity of urine in HIV-1 infected and HIV negative healthy individuals showed significant difference **(*P* value 0.003)**

All 139 samples from HIV infected individuals and 83 healthy control samples which were negative for BK virus DNA were subjected to in house ERV-3 Quantitative real time PCR to check the DNA integrity of the samples. All the samples from 222 individuals were positive for ERV-3.

The representative real time amplification plot for BKV DNA is shown in **Figure 14**

Figure 14: Representative real time amplification plot for quantitation of BKV DNA



Legend:

QS-1- Quantitation standard for BKV DNA (10^6 Copies/ml)

QS-2- Quantitation standard for BKV DNA (10^5Copies/ml)

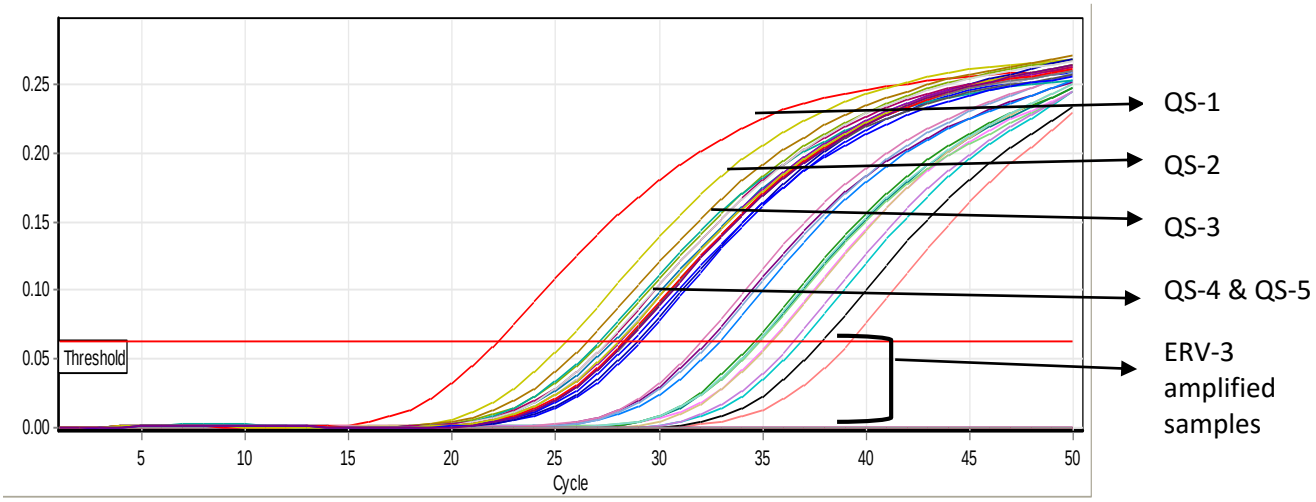
QS-3- Quantitation standard for BKV DNA (10^4 Copies/ml)

QS-4- Quantitation standard for BKV DNA (10^3 Copies/ml)

QS-5- Quantitation standard for BKV DNA (10^2 Copies/ml)

The representative real time amplification plot for ERV-3 is shown in **Figure 15**

Figure 15: Representative real time amplification plot for quantitation of ERV-3



Legend

QS-1- Quantitation standard for ERV-3 (10^6 Copies/ml)

QS-2- Quantitation standard for ERV-3 (10^5Copies/ml)

QS-3- Quantitation standard for ERV-3 (10^4 Copies/ml)

QS-4- Quantitation standard for ERV-3 (10^3 Copies/ml)

QS-5- Quantitation standard for ERV-3 (10^2 Copies/ml)

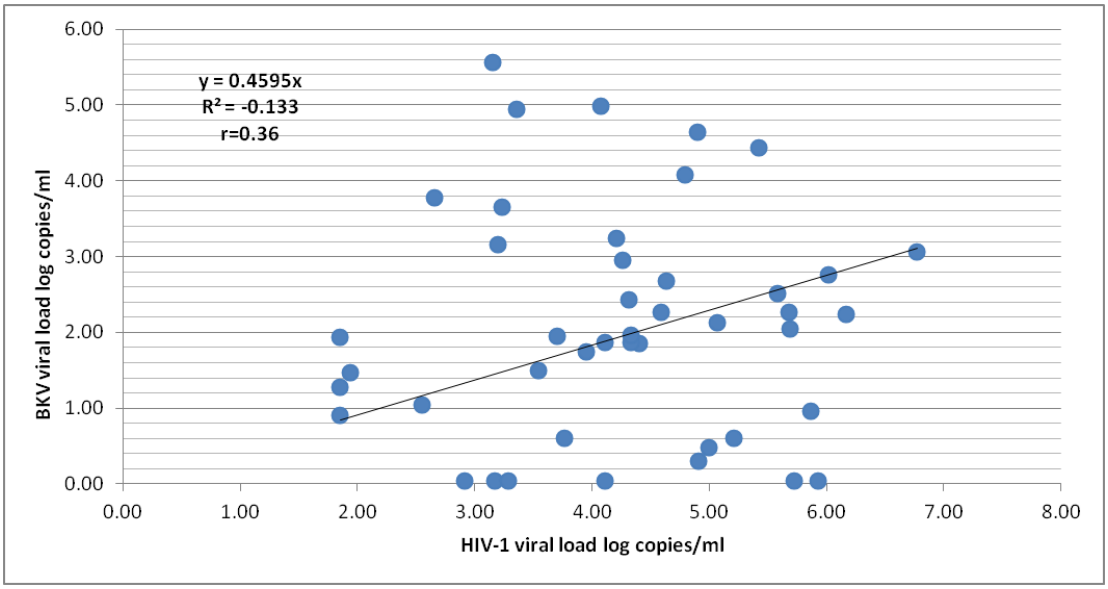
5.6.1 Association of BK viral load and HIV-1 viral load

Regression plot analysis of BKV load and HIV load in log copies in BKV DNA positive HIV-1 infected individuals did not show the best agreement (r=0.36, n=45) (*P* value 0.37)

5.6.2 Comparison of BKV DNA load between HIV-1 infected individuals and healthy controls

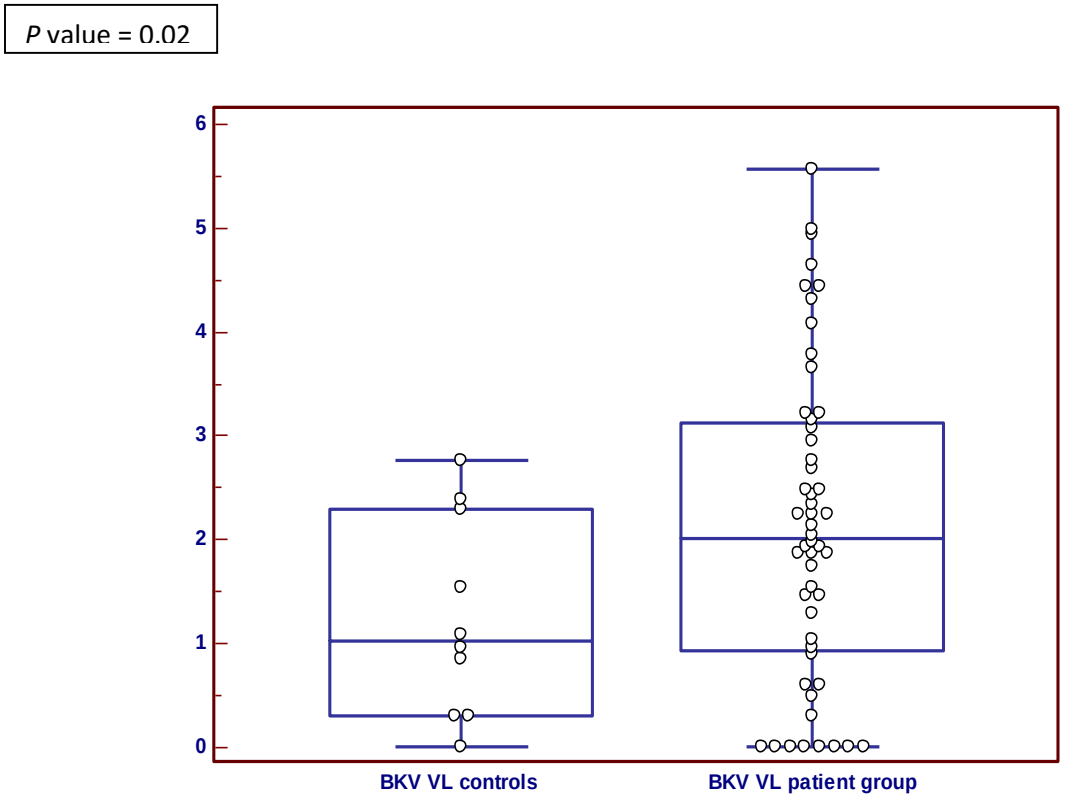
When BKV DNA load of HIV-1 infected individuals were compared with that of HIV negative healthy individuals, there was a significant difference with a ***P* value of 0.02.** The regression plot representing the comparison of BK viral load with HIV viral load in BKV DNA positive HIV1 infected individuals is shown in **Figure 16.**

Figure 16: Comparison of BKV load and HIV-1 load in BKV DNA positive treatment naïve HIV-1 infected individuals using regression plot analysis



The box and whisker plot showing the BKV load comparison between HIV-1 infected individuals and healthy controls is shown in **Figure 17**.

Figure 17: Box and whisker plot for comparison of BKV load in HIV-1 infected individuals and HIV-1 negative healthy controls



5.6.3 BK positivity and WHO staging

Among the total 115 HIV-1 infected individuals under WHO clinical stage 1, 24 (20.8%) individuals showed detectable BKV DNA - 22 (19%) in urine and 2 (1.7%) in whole blood. The BKV load in this category range from 1-87368 copies/ml. Of 16 individuals in stage

2, 3 (18.8%) individuals showed BKV DNA positivity with viral load range from 3-359886 copies/ml. 7 (24%) out of 29 subjects in clinical stage 3 were positive for BKV DNA with viral load ranging from 8-95645 copies/ml and 14 (51.8%) out of 27 individuals in stage 4 had BKV DNA in their urine samples with viral load 1-27837 copies/ml.

The percentage positivity and mean BK load of HIV-1 infected individuals is represented in **Table 26**

BKV load observed in the asymptomatic stage 1 and that of symptomatic stages (2, 3, and 4) showed significant difference in the viral load (***P* value 0.005)** with higher viral load in the symptomatic group.

Table 26: BKV positivity and their mean viral load of HIV-1 infected individuals categorized based on WHO clinical stages

BKV DNA Positivity and mean viral load	**WHO staging**			
	1 (n=115)	2 (n=16)	3 (n=29)	4 (n=27)
Positive samples	24	3	7	14
Percentage positivity	20.8%	18.8%	24%	51.8%
Mean log $_{10}$ copies ± SD	1.70±1.41*	(2.82±1.56	3.03±1.68	2.32±1.23)*

****** *BKV load observed in the asymptomatic stage 1 and that of symptomatic stages (2, 3, and 4) showed significant difference in the viral load (**P* **value 0.005*)** with higher viral load in the symptomatic group.*

One way ANOVA analysis showed that there was a significant difference in mean of BK viral load among the four WHO clinical stages of HIV. **(*P* value < 0.001)**

5.6.4 Comparison of BK viral load with different variables

The mean BK viral load was compared with different variables such as CD4 count (350 cells/µl as the cut off), HIV-1 viral load and WHO staging was done as shown in **Table 27**

Table 27: Comparison of mean and SD between HIV-1 and BK viral load based on the CD4 counts and WHO staging among HIV-1 infected individuals.

CD4 count	BKV load range (copies/ml)	Mean log_{10}±SD	HIV-1 load range (copies/ml)	Mean log_{10}±SD	WHO stage
<350 (25)	1-26882	2.04±1.16	71-5888659	4.69±1.23	Stage 4(n=13) Stage 3(n=3) Stage 2(n=1) Stage 1(n=8)
>350 **(21)**	1-359886	2.82±1.32	71-489718	3.73±1.10	Stage 1(n=15) Stage 2(n=2) Stage 3(n=4)

The univariate analysis of BK viral load outcome showed significant negative correlation with CD4+ T cell counts (p value 0.026) and a positive correlation with WHO clinical staging (p value 0.002). The variables which were significant by univariate analysis were subjected for multivariate analysis. This BK viral load multivariate analysis showed significant correlation only with WHO clinical staging (*P* value 0.007) and no significance with CD4 counts (*P* value 0.64). The univariate and multivariate analysis of CD4 cell count and WHO staging with BK viral load as outcome is represented in **Table 28**

Table 28: Univariate and multivariate analysis of CD4 cell count and WHO staging with BK viral load as outcome

Variable	**N**	**Cut off**	**Univariate analysis**	**Multivariate analysis**		
			***p* value**			
				Odds ratio	95% CI	*P* value
Age	187	40	0.91	NI	NI	NI
Sex	187	-	0.25	NI	NI	NI
CD4 counts	186	350	**0.02**	0.81	0.3	0.64
HIV-1 viral load	102	-	0.49	NI	NI	NI
WHO staging	187	-	**0.00**	1.60	1.1	**0.00**

NI – Not included

5.7 Findings on follow up samples from HIV-1 infected individuals after 3-6 months of ART

Among the 74 HIV-1 infected individuals who had CD4 counts <350 cells/µl, Eight individuals who were BKV DNA positive and who came to the Department of Clinical Virology for CD4 counts were followed up after a minimum of 3 months of ART. These patients. The mean BK viral load of these individuals before starting ART was 3664 copies/ml. Among 8 subjects, 4 were negative for BKV DNA, 2 had higher and the remaining 2 had lower BK viral load compared to their pre ART BK viral load levels. Thus 6 (75%) out of 8 individuals showed an effective response of either decrease in BK viral load (n=2, 25%) or negativity for BK virus (n=4, 50%) with ART as represented in **Figure 18.**

The CD4 counts and BK viral load were compared before and after taking ART as shown in **Table 29.**

Comparison of BKV viral load in log copies/ml in pre and post ART individuals using Paired T test showed no statistically significant difference in the viral load. (*P* value 0.24).

There was no significant difference between pre ART and post ART BKV mean viral load (*P* value 0.24). This is represented as a box and whisker plot as shown in **Figure 19**

Table 29: CD4 counts and BK viral load values before and after ART in follow up subjects

S.No	Pre ART values		Post ART values	
	CD4 count (cells/µl)	**BK viral load (copies/ml)**	**CD4 count (cells/µl)**	**BK viral load (copies/ml)**
1.	362	1	590	0
2.	261	1	551	0
3,	133	9	300	34
4.	222	88	534	0
5.	46	582	404	20428
6.	72	26882	297	297
7.	312	134	807	0
8.	45	1615	115	219

Comparison of BKV viral load in log copies/ml in pre and post ART individuals using Paired T test showed no statistically significant difference in the viral load. (*P* value 0.24)

Figure 18: Pie chart showing the BKV outcome in follow up individuals

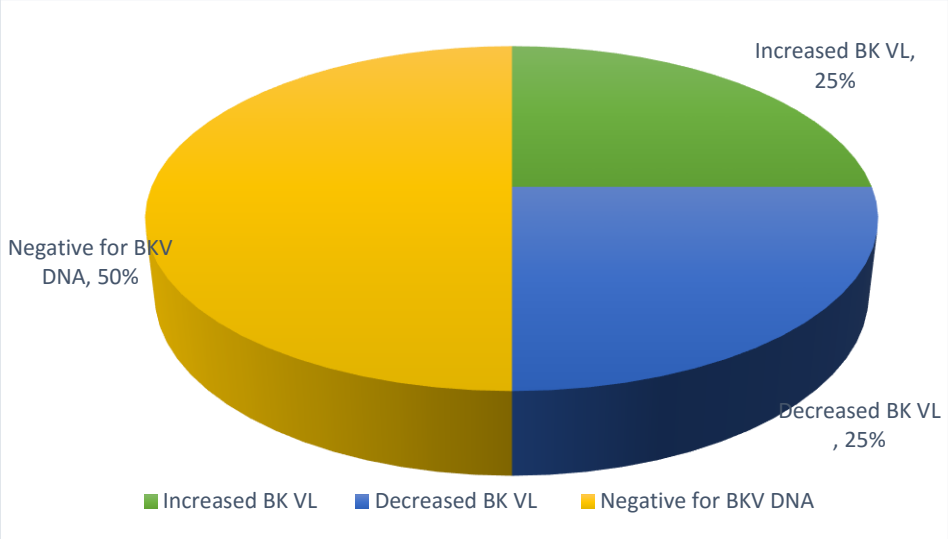
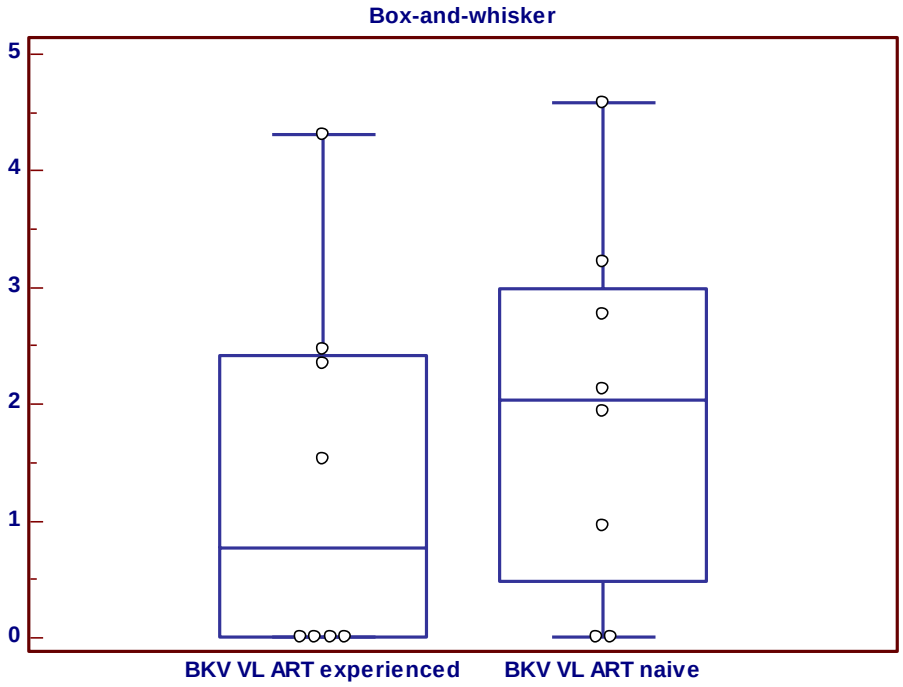


Figure 19: Box and whisker plot for comparison of BKV mean viral load before and after ART in HIV-1 infected individuals

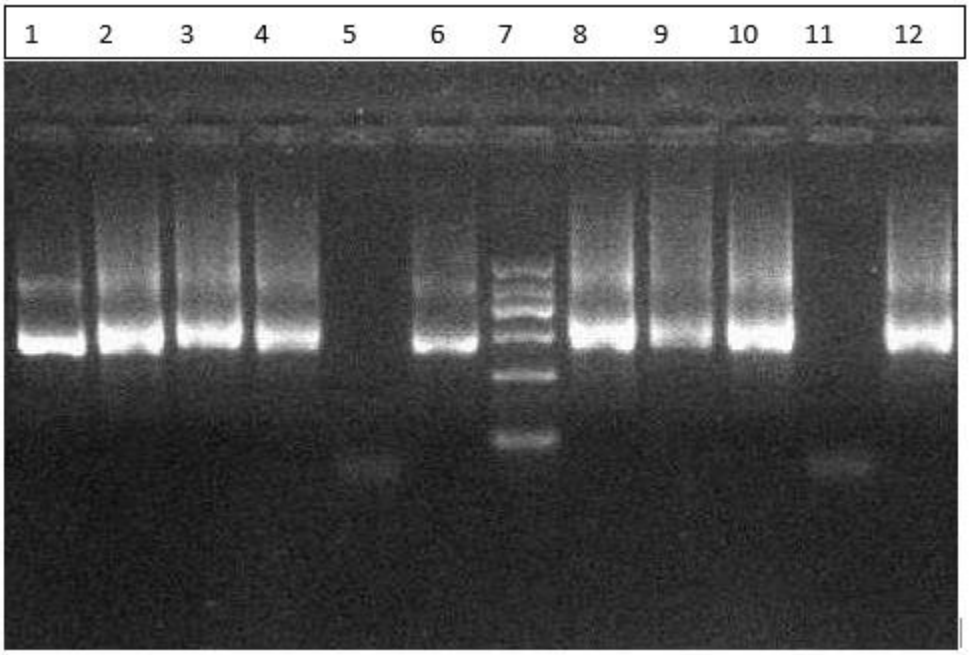
P value = 0.24



5.8 NCCR sequencing

A proportion of BKV DNA positive samples were sequenced using Sanger sequencing method to determine any rearrangements in the BKV NCCR region**.** Out of 46 BKV DNA positive urine samples, 32 were sequenced. 2 whole blood samples which were positive for BKV DNA were negative for NCCR PCR even after repeated testing**.** To look for any rearrangements in blood, two whole blood archive samples positive for BKV in HIV-1 infected individuals from our previous study were also sequenced. NCCR sequencing was also performed on BKV DNA positive urine samples from follow up individuals (n=4) and healthy subjects (n=5). All BKV nucleotide sequences showed good quality electropherogram and were analysed using finch TV software. The solved sequences were aligned with the reference archetype NCCR sequences using BioEdit software version 7.2.5. The representative gel picture showing BKV NCCR amplified products is given in **Figure 20.** The representative electropherogram showing NCCR sequences and VP1 sequences are shown in **Figure 21 and Figure 22.** The aminoacid alignment of BKV NCCR study samples using BioEdit software is shown in **Figure 22.**

Figure 20: Representative gel documentation picture showing the BKV NCCR amplified products



Legend:

Lane 1-4, 6 & 8-10 – BKV NCCR amplified products – product size 233bp

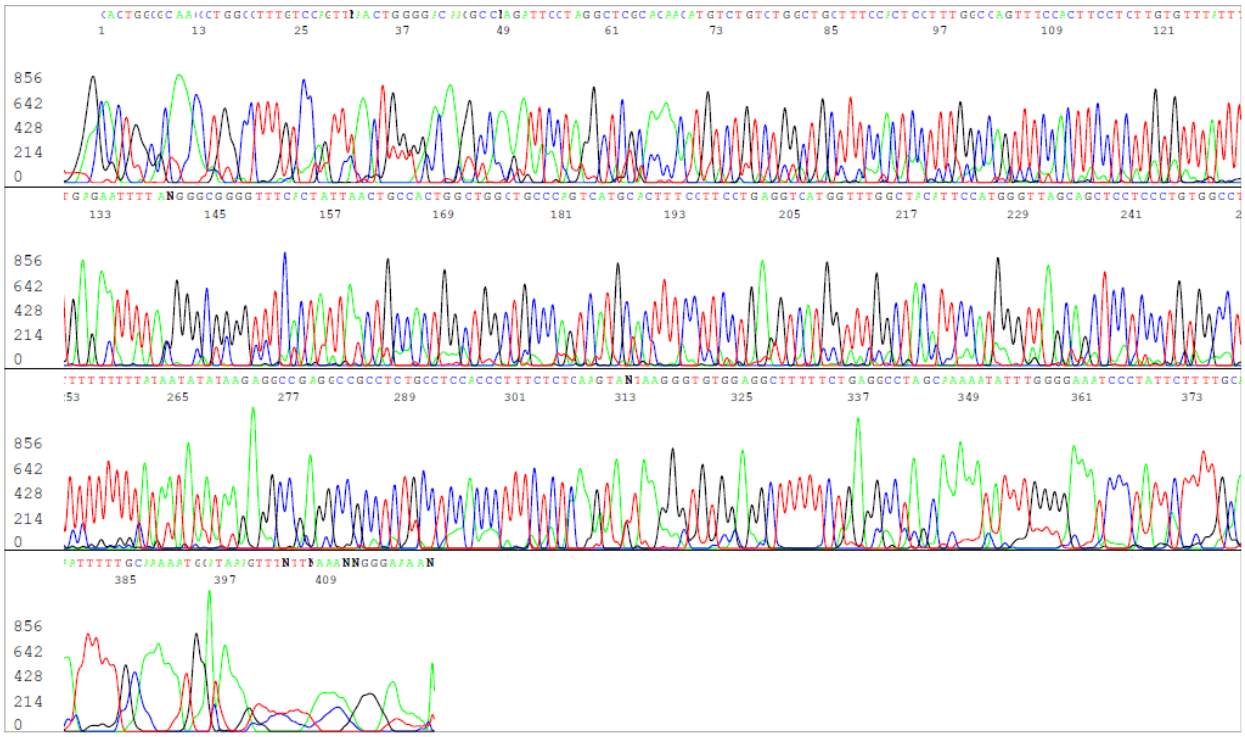
Lane 7 - Molecular weight ladder (100-1200bp) (Bio basic Inc, Ontario, Canada)

Lane 12 – Positive control

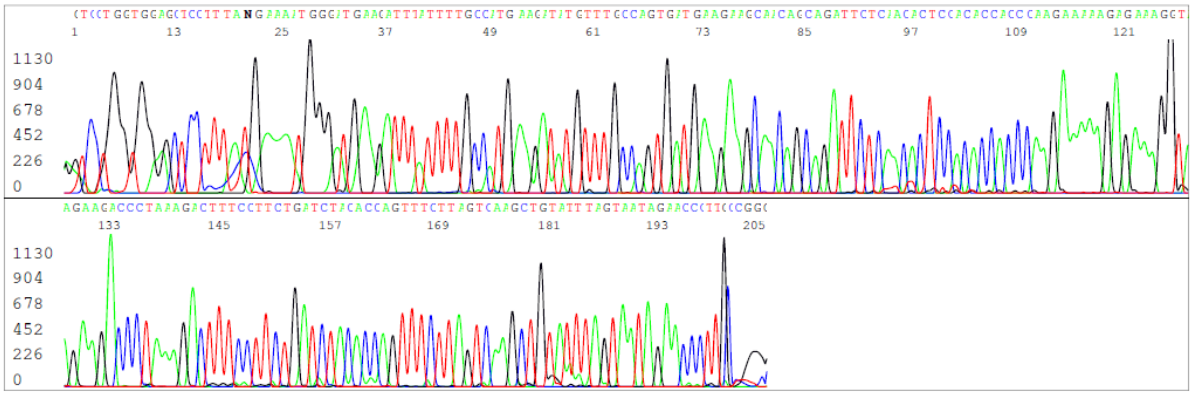
Lane 5 & 11 – Negative control

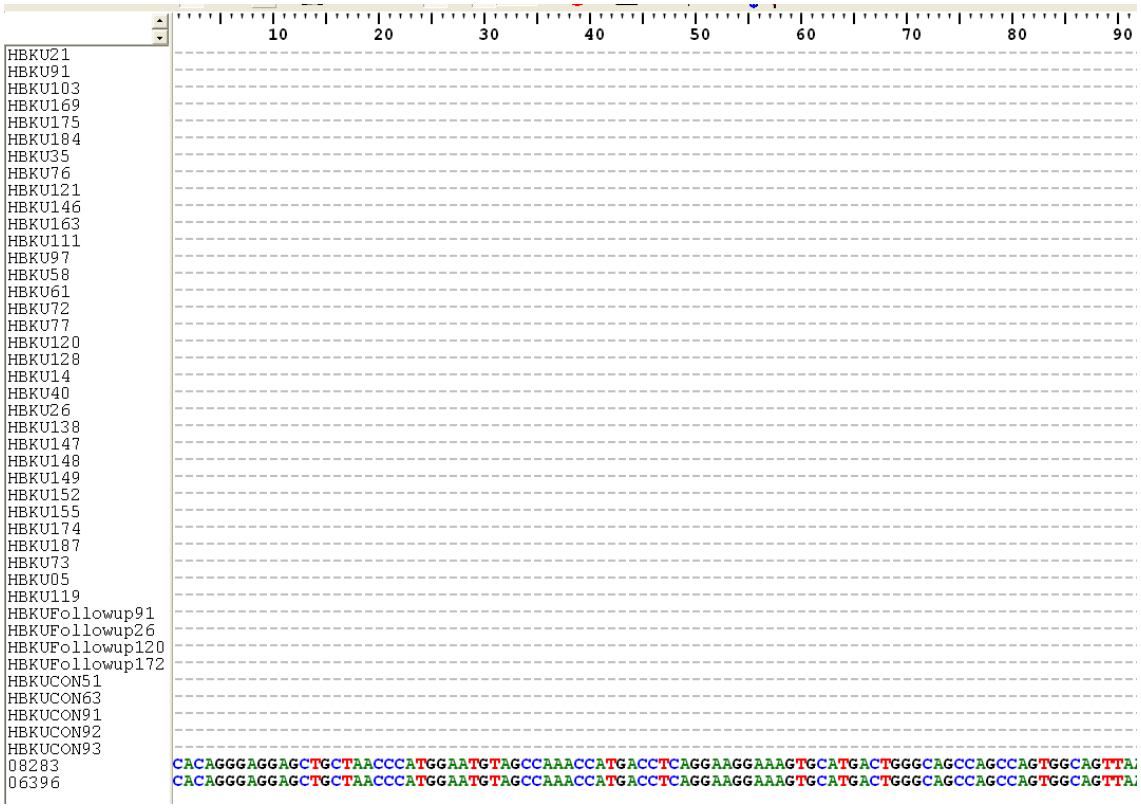
Figure 21: Representative electropherogram showing NCCR and VP1 sequences of BK virus

(a) NCCR sequences



(b) VP1 sequences




10 20 30 40 50 60 70 80 90
HBKU21
HBKU91
HBKU103
HBKU169
HBKU175
HBKU184
HBKU35
HBKU76
HBKU121
HBKU146
HBKU163
HBKU111
HBKU97
HBKU58
HBKU61
HBKU72
HBKU77
HBKU120
HBKU128
HBKU14
HBKU40
HBKU26
HBKU138
HBKU147
HBKU148
HBKU149
HBKU152
HBKU155
HBKU174
HBKU187
HBKU73
HBKU05
HBKU119
HBKUFollowup91
HBKUFollowup26
HBKUFollowup120
HBKUFollowup172
HBKUCON51
HBKUCON63
HBKUCON91
HBKUCON92
HBKUCON93
08283 CACAGGGAGGAGCTGCTAACCCATGGAATGTAGCCAAACCATGACCTCAGGAAGGAAAGTGCATGACTGGGCAGCCAGCCAGTGGCAGTTA
06396 CACAGGGAGGAGCTGCTAACCCATGGAATGTAGCCAAACCATGACCTCAGGAAGGAAAGTGCATGACTGGGCAGCCAGCCAGTGGCAGTTA


90 100 110 120 130 140 150 160 170 180
HBKU21 CACAGGGAGGAGCTGCTAACCCATGGAATGTAGCCAAACCATGACCTCAGGAAGGAAAGTGCATGACTGGGCAGCCAGCCAGTG
HBKU72 T
HBKU128 T
HBKU187 T
HBKU73 T
08283 TAATAGTGAAAC.CC
06396 TAATAGT

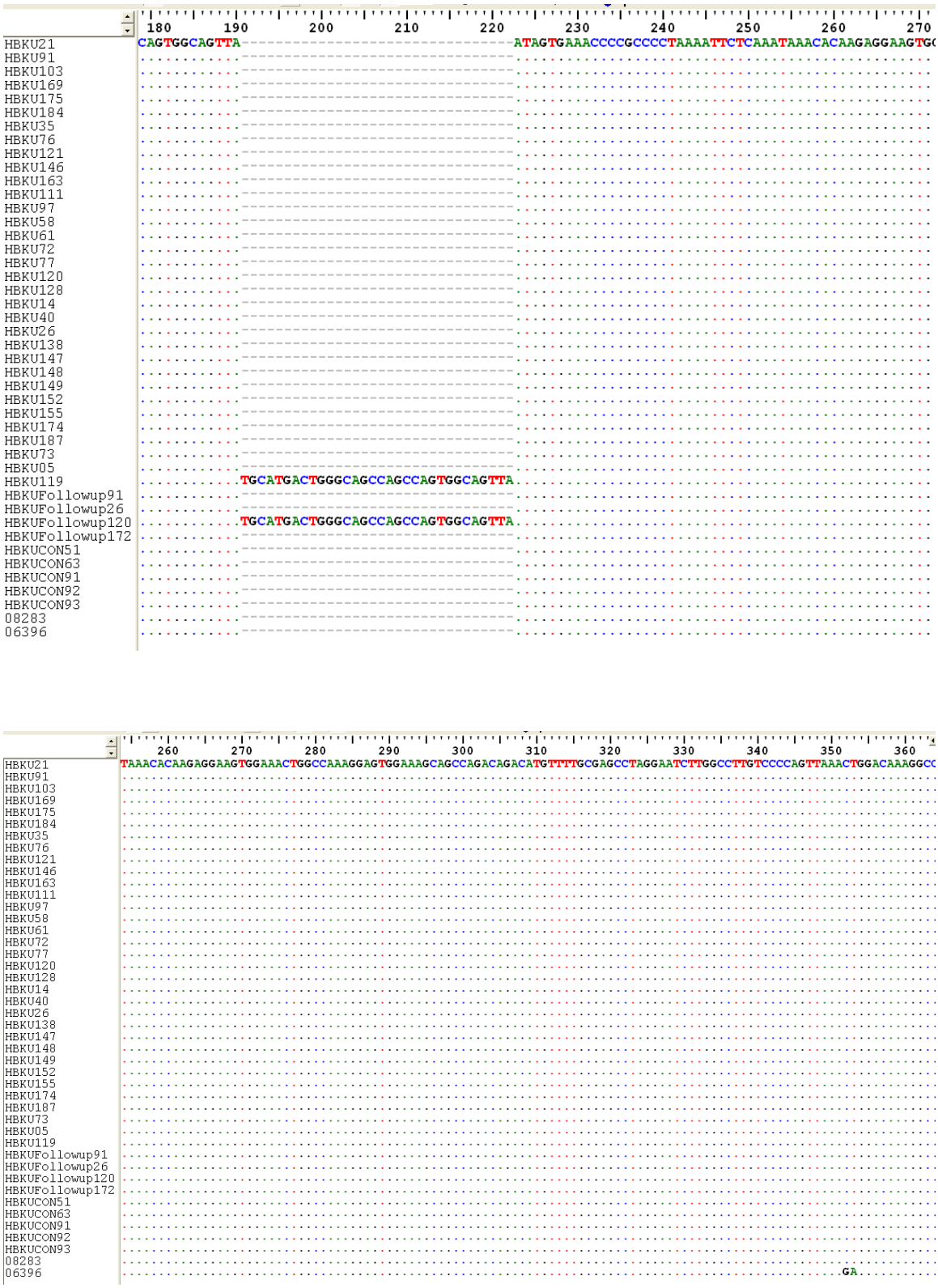


Figure 22: The amino acid alignment of BKV NCCR study samples. The entire consensus sequences were aligned using Bio Edit software. All positions that agree with the consensus sequence are denoted by a dot (.).

The archetype NCCR consists of 4 sequence blocks namely P block (68bp), Q (39bp), R (63bp), and S block (63bp). The number in the brackets indicate the number f nucleotides in each block of NCCR. Two urine samples, one from a treatment naïve HIV-1 infected individual and the other from a follow up subject showed rearrangements in the NCCR region. Both the archived whole blood samples also showed NCCR rearrangements. The rearrangement pattern seen in our clinical strains comprised of duplication of a part of P (9bp) and Q (23bp) blocks of BKV NCCR. The rearrangement seen in archived blood sample showed duplication of almost the whole of P (60bp) and Q (35bp) blocks in one sample and duplication of a part of P (51bp) and Q (28bp) blocks in the other sample.

Figure 23: Representation of archetype and rearranged BKV NCCR region observed in various study samples

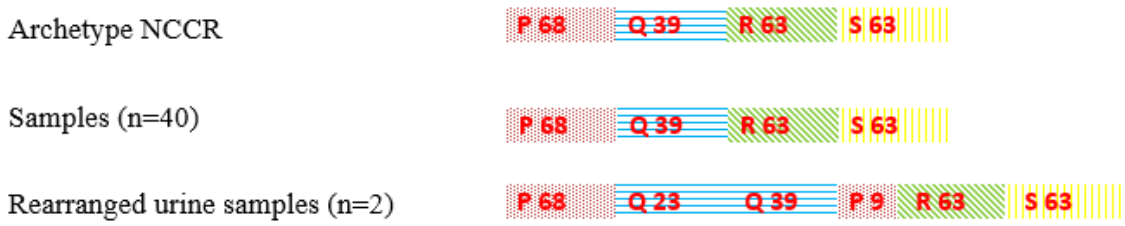
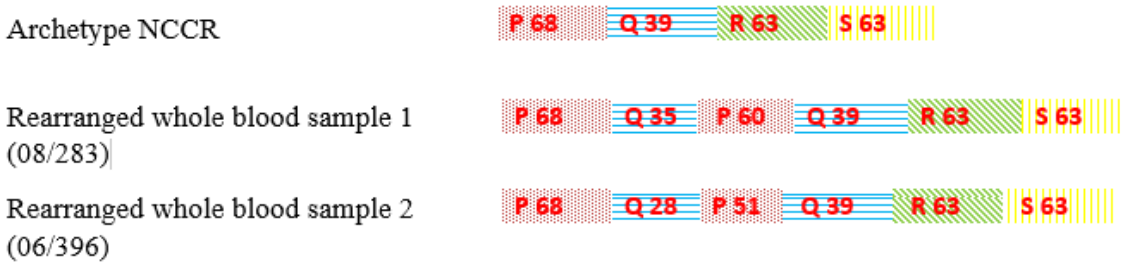


Figure 24: Representation of rearranged BKV NCCR in archived blood samples



The representation of the archetype and rearranged NCCR region in various study samples is shown in **Figure 23.** The rearrangements in NCCR region seen in archived blood samples is represented in **Figure 24.** The CD4 count, BKV load and HIV load of NCCR sequenced study samples and archived samples were compared and this is shown in **Table 30**

Table 30: Details of CD4 counts, HIV-1 and BKV load in NCCR sequenced study and archived samples

	NCCR	**BKV load range (copies/ml)**	**CD4 counts (cells/µl)**	**HIV load (copies/ml)**
Archetype	**Samples (n=32)**	11-359886 (2.81±1.18)	18-697	71-5888659 (4.25±1.27)
	Follow up (n=3)	34-20428 (2.66±.17)	115-807	-
	Controls (n=5)	12-572 (1.24±0.96)	-	-
Rearranged	**Urine sample 1**	29	697	87
	Urine sample (follow up)	72	297	-
	Archived Blood sample 1	17200	38	795041
	Archived Blood sample 2	3000	6	266921

Though the samples showed the rearranged pattern of NCCR there was no association between the rearrangement and BKV viral load as the two samples showed very low BK copy number.

5.9 BKV genotyping

Out of the total 46 BKV DNA positive urine samples, 30 were genotyped based on NCCR gene analysis and randomly selected 5 samples were genotyped based on VP1 gene sequencing. Two blood samples which were positive for BKV DNA were negative for genotyping even after repeated testing**.** Genotyping was also performed on follow up samples (n=8) and BKV positive control samples (n=10). Two archived blood samples which were positive for BKV from a previous study done in our institution were also genotyped. All BKV nucleotide sequences showed good quality electropherogram and were analysed using finch TV software. The BKV genotypes were determined based on the phylogenetic analysis of BKV strains from the Gen bank reference sequences (BKV genotype reference numbers are as follows: FM995410 (I), DQ176632 (II), AB211386 (III), FM995460 (IV)). All samples (n=48, 100%) analysed were found to be BKV genotype I. The five samples genotyped based on VP1 were also identified as genotype I.

All the samples sequenced for NCCR were submitted to Gen Bank database and obtained accession numbers KT728853 - KT728896.

The phylogenetic tree showing the BKV genotypes of study samples based on NCCR gene analysis is shown in **Figure 25**

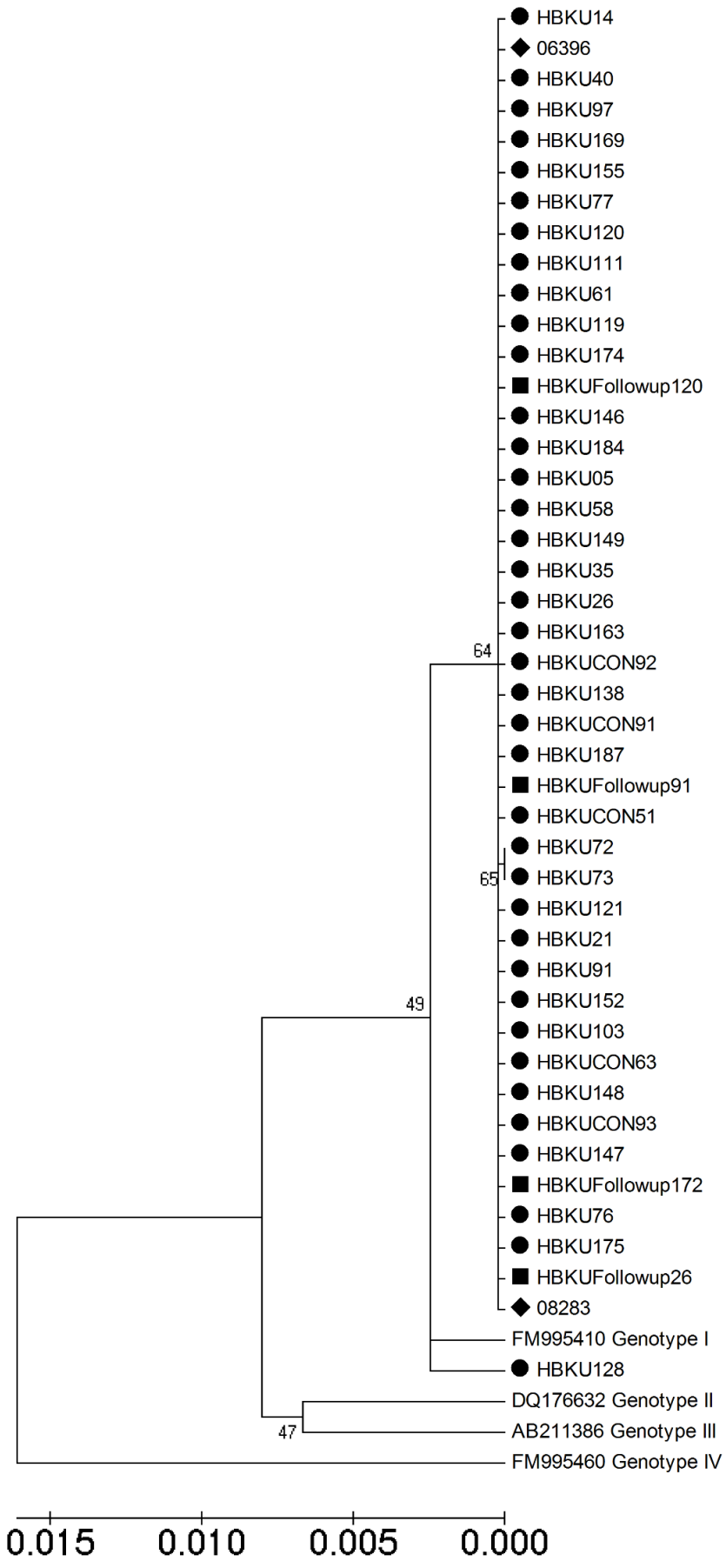

HBKU14
06396
HBKU40
HBKU97
HBKU169
HBKU155
HBKU77
HBKU120
HBKU111
HBKU61
HBKU119
HBKU174
HBKUFollowup120
HBKU146
HBKU184
HBKU05
HBKU58
HBKU149
HBKU35
HBKU26
HBKU163
64
HBKUCON92
HBKU138
HBKUCON91
HBKU187
HBKUFollowup91
HBKUCON51
HBKU72
65
HBKU73
HBKU121
HBKU21
HBKU91
49
HBKU152
HBKU103
HBKUCON63
HBKU148
HBKUCON93
HBKU147
HBKUFollowup172
HBKU76
HBKU175
HBKUFollowup26
08283
FM995410 Genotype I
HBKU128
DQ176632 Genotype II
47
AB211386 Genotype III
FM995460 Genotype IV
0.015
0.010
0.005
0.000

Figure 25: Phylogenetic tree to determine BKV NCCR genotypes in patient population. Reference sequences from GenBank database are identified with accession numbers. Urine samples sequences are shown with shaded circles (●), whole blood samples are represented with shaded diamond shaped icon (♦) and follow up samples with shaded squares (■). The phylogenetic analysis was performed in MEGA4 software using the neighbor joining method with a bootstrap test of 1000 replicates. The accession numbers of the reference sequences for BKV genotypes are as follows FM995410 (I), DQ176632 (II), AB211386 (III), FM995460 (IV)

The phylogenetic tree showing the BKV genotypes of study samples based on VP1 gene analysis is shown in **Figure 26**

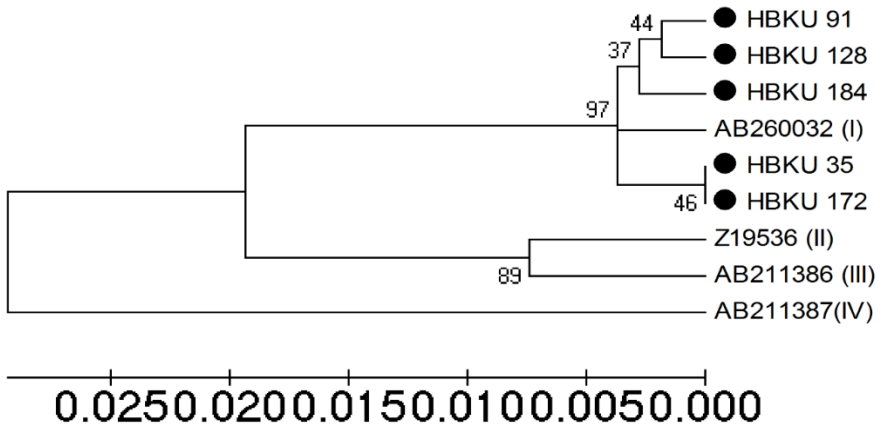


Figure 26: Phylogenetic tree to determine BKV VP1 genotypes in randomly selected patient population. Reference sequences from GenBank database are identified with accession numbers. Urine samples sequences are shown with shaded circles (●). The phylogenetic analysis was performed in MEGA4 software using the neighbor joining method with a bootstrap test of 1000 replicates. The accession numbers of the reference sequences for BKV genotypes are as follows AB260032 (I), Z19536 (II), AB211386 (III), AB211387 (IV)

.6. Discussion

Human Immunodeficiency Virus infection has been a formidable threat to mankind since its discovery. At the end of 2014, there were about 36.9 million people living with HIV worldwide claiming about 1.2 million lives last year (1). Among the two types of HIV, HIV-1 is more common globally causing severe spectrum of disease than HIV-2 (5). HIV-1 is transmitted mainly by parenteral (contact with infected body fluids and unsafe injection practices), sexual and mother to child transmission (3). The virus gains entry into the body through specific receptors on its target cells namely, CD4+ T helper cells and macrophages. As it targets the immune cells, the virus causes severe immunosuppression leading to various infections which are collectively called as opportunistic infections (6). Apart from the defined opportunistic illnesses, any infectious agent can cause significant disease in HIV infected individuals attributing to the severe immunosuppression produced by HIV (59). The infection can either be a primary infection or reactivation of the infectious agent especially a virus from a latent site causing significant morbidity and mortality (8).

The incidence of opportunistic infections in western countries has reduced to a large extent owing to the outreach of ART in those regions. Whereas in our country, these infections still remain an important health issue because of the resource constraints and lack of proper reach of ART services (103). As of 2011, there are 20.9 lakhs people living with HIV in India. Of them 7,68,840 people were on ART at the end of 2014

(104). The most common opportunistic infections among HIV infected individuals in India are tuberculosis and candidiasis accounting for about 64% and 58% respectively followed by other infections (105).Viruses are an important cause of opportunistic infections in HIV infected individuals. The major viral agents are Cytomegalovirus (CMV) causing retinitis, Epstein Barr Virus (EBV) causing lymphoma, John Cunningham Virus (JC virus) causing Progressive multifocal leucoencephalopathy, Herpes simplex and Herpes zoster infections. A study done from our institution by Sachithanandham *et al* (2009), had reported the prevalence of various opportunistic viruses in our part of the country. The viruses looked for in that study were CMV, HSV-1 & 2, EBV, VZV (Varicella zoster virus), *Adenovirus*, JC and BK virus. The authors reported 68 whole blood samples from HIV infected individuals were tested using multiplex PCR and 72% positivity for EBV and 7% positivity for CMV was observed (106).

Another study by the same group in 2014 where the frequency of various opportunistic DNA viruses in treatment naïve HIV-1 infected individuals had been studied. They showed a positivity for EBV in 82% and 73% in patients who had CD4 counts <100cells/µl and 350cells/µl respectively. CMV DNA was found in 29.6% and 2.8% of whole blood samples from the two groups of patients who were segregated based on their CD4 counts. They also found 2 BK and 2 JC virus positive whole blood samples in that study (101). Thus BK virus is an important opportunistic viral agent to be looked at in immunocompromised individuals. It is a DNA virus that belongs to the family

Polyomaviridae (9).The disease occurs due to reactivation of the virus from its latent site mainly the kidneys. The virus causes significant nephropathy in immunosuppressive states frequently reported in renal transplant recipients who are on immunosuppressive therapy (15).Following reactivation, there is rapid multiplication of the virus causing three major clinical scenarios namely BKV associated nephropathy seen commonly in renal transplant patients, late onset hemorrhagic cystitis in hematopoietic bone marrow transplant recipients and ureteric stenosis (17). Rarely case reports of meningoencephalitis, retinitis and pneumonia have been reported to be caused by BK virus especially in HIV infected individuals (17–19). BK viruria occurs in 80% of the renal transplant patients with 5-10% patients progressing to nephropathy after 3 months of transplantation (86).

As HIV is also an immunodeficient state where there is severe depletion of CD4+ T cells, it is important to look for this virus in these individuals. But there are limited studies on the impact of BK virus in HIV. The study reported in this thesis had estimated the viral load burden of BK virus from urine and whole blood among HIV-1 infected individuals using a well characterized and evaluated in house quantitative real time PCR and correlating with the extent of immunosuppression. We also determined the effect of ART on clearance of BK virus without any BKV specific treatment. Out of the 187 treatment naïve HIV-1 infected individuals, urine samples from 46 (25.6%) individuals were found to be BKV positive which is almost the same (24%) as reported by Sundsfjord *et al* (1994) where 20 out of 82 HIV infected individuals were found to

be BKV DNA positive by PCR from urine samples (107). However this when compared to Ledesma *et al* (2012), study where 45 (57.7%) out of 78 urine samples collected from HIV infected individuals had BK viruria with viral load less than 100 copies/ml. In this group of patients, only 5 were treatment naïve individuals of which 3 (60%) were positive for BK virus detected by nested PCR (14). But we observed low frequency of BK viruria in treatment naïve HIV-1 infected individuals with 21 individuals showing BK viral load >100 copies/ml.

The same group found only 1 (1.2%) plasma sample positive for BKV DNA from an HIV infected individual out of the total 78 individuals (14). There are other studies that showed higher frequency of BKV DNA in plasma and PBMC samples from HIV infected individuals. A study from Iran, done by Akhgari S *et al* (2015), showed 16% (4/25) BK viremia among treatment naïve HIV infected individuals by nested PCR (108). Another study from Norway, has reported 4.8% (2/42) positivity in PBMC samples of HIV infected individuals (107). In our study, BKV DNA was detected in only 2 out of 187 (1%) whole blood samples from HIV-1 infected individuals each with a viral load of 1 copy/ml even after testing twice. This emphasizes the reproducibility of our real time PCR for BK virus at low copy numbers. The frequency of viremia (1%) observed in the study mentioned in the thesis is almost the same as Ledesma *et al* (2012) (1.2%) but lower than the other two studies. However, when compared to these studies the sample matrix and treatment status were different from our study. But as mentioned before, Sachithanandham *et al* (2014), who used whole blood samples from treatment

naïve HIV-1 infected individuals, 2 (0.6%) individuals were positive for BK virus with higher BK viral load (3000 and 17200 copies/ml) (101).

In renal transplant settings, the BKV associated disease starts with viruria and with unrestricted multiplication due to immunosuppression leads to viral shedding in plasma (86). The frequency of BK viremia has been reported to be about 20% after 1 year of transplantation (72). Viremia is considered to be a predicting factor for the development of nephropathy which occurs usually 1-12 weeks post-transplant finally leading to allograft failure (84) This increased shedding of virus in blood and associated nephropathy very rarely occurs in immunocompromised states other than renal transplantation. This emphasizes the role of various factors associated with transplantation in the development of nephropathy (54). In the above mentioned studies as well as our study, the frequency of BK viremia range from 1-5% which is much lower than seen in transplant settings.

Akhgari S *et al* (2015) has observed 7 out of 8 HIV infected individuals who had BK viremia to have CD4 counts >200 cells/µl (108). The BK viremia detected in one individual by Ledesma *et al* (2012), had a high CD4 count of 880cells/mm^3. The CD4 counts of two patients who showed BK viremia in our study also had CD4 counts >200 cells/µl (327 and 667 cells/µl). The association between CD4 counts and frequency of viruria or BK viral load remains controversial. Akhgari S *et al* (2015) and Sundsfjord *et al* (1994) have reported that BK viruria is independent of CD4 counts (107,108).

Another study from Italy, Di Taranto *et al* (1997), where of the 33 HIV-1 infected children whose age group ranging from 3 to 7 years studied, 2 (6%) children had BK viruria with no correlation between CD4 counts and viruria or viral load (109). On the other hand, Jin *et al* (1995), from UK studied 101 HIV individuals, of which 45 patients were positive for BK virus in their urine. The highest proportion of this positive subset had CD4 count <200 cells/µl. So this study emphasizes that the frequency of BK viruria is proportional to the degree of immunosuppression (110). Sachithanandham *et al* (2014) who reported 2 BK viremic patients, found that both patients had CD4 counts < 100cells/µl (6 and 38 cells/ µl) (101). However Ledesma *et al* (2012) had reported higher BKV positivity in patients with CD4 counts >500 cells/µl (14). In our study, there was a significant negative association between low CD4 counts and BKV positivity (*P* value 0.02).

The association between HIV load and BKV positivity observed by Ledesma *et al*, 2012 (*P* value 0.51) was statistically insignificant. But the author mentions about three treatment naïve urine BKV DNA positive HIV infected individuals, who had higher HIV viral load as well as BKV DNA concentration (14). According to the data retrieved from Sachithanandham *et al* (2014), the HIV load of the two ART naïve BK viremic individuals were higher (795041 and 266921copies/ml) emphasizing the correlation between the degree of immunosuppression and the presence of BK virus in blood (101). In the present study, when we compared the urine BKV load with HIV load (only BKV DNA positive individuals), there was no significant correlation (*P* value 0.37).

According to WHO, clinical staging of HIV range from asymptomatic stage to a severe stage, known as AIDS in which the individual has defined opportunistic infections. Behzad Behbahani *et al* (2004) has reported that 12 out of 19 (63.1%) BK positive HIV patients belonged to AIDS (stage 4) and the rest were asymptomatic (111). Few studies have staged their patients based on CDC classification of HIV where infected individuals are categorized into 3 groups based on CD4 count and clinical conditions at the time of presentation. CD4 counts > 500 cells/µl as category 1, 200-499 cells/µl as category 2 and <200 cells/µl as category 3 (38). According to Di Taranto *et al* (1997), 2 BKV positive children belonged to CD4 category 1 (> 500 cells/µl) (109). Similar findings were reported by Ledesma *et al* (2012) where 26 out of 45 BKV positive HIV patients had CD4 counts > 500 cells/µl (14). Jin *et al* (1995) reported higher number of BKV positives in CD4 category 3 (<200 cells/µl) (110). Akhgari S *et al* (2015), of the total 99 HIV infected individuals, 30 (30.3%) patients were under category 1, 50 (50.5%) were under category 2 and 19(19.2%) were under CD4 category 3. Out of the 8 BKV DNA positive patients, viremia was found to be higher in 7 (87.5%) subjects who were either under category 1 or 2 (≥ 200 cells/µl) (108). In our study, WHO staging when compared with the BKV positivity and viral load, significant difference among the four stages of HIV (*P* value <0.001) was observed with BKV percentage positivity being highest (51.8%) in stage 4. According to CDC classification, 101 patients were under category 1, 42 under category 2 and 43 subjects under category 3. Among the urine BKV positive patients, majority (71.7%) were under category 2 or 3 with equal distribution within the two categories. Even though the highest percentage positivity of BK virus in HIV infected individuals was seen

in WHO stage 4 or CDC stage 3, none of the individuals had any clinical symptoms suggestive of BKV nephropathy.

The effect of HAART on opportunistic infections in general is like a double edged sword. The frequency of these infections is much higher in treatment naïve HIV infected individuals than ART experienced group (105). This is because of the restoration of the immune system by improving the CD4 counts on ART. On the other hand, with the initiation of ART, the scenario of IRIS (Immune Reconstitution Inflammatory Syndrome) sets in which makes the patient vulnerable to various opportunistic infections within the first three months (105). With respect to BKV infection, reduction of immunosuppression plays a major role in transplant settings (22). In case of HIV, HAART has a tremendous effect in improving the immunosuppression even without any BK specific intervention (23).

Vidal *et al* (2007) had reported a case of a BKV associated meningoencephalitis who had improved just with ART and without any BK specific treatment. Multiplex PCR was performed on CSF for all human herpes viruses, JC and BK viruses. PCR was done on brain biopsy tissue as well and BK was confirmed. Patient was started on HAART and there was an improvement in CD4 count from 6 to 144 cells/µl at the end of 5 months with regression of the brain lesions on MRI even though the patient had IRIS at that presentation. But IRIS improved with the continuation of ART and the patient recovered (112). Ledesma *et al*, 2012 had observed that treatment naïve individuals had higher BKV as well as HIV load when compared to individuals on treatment. In that study, of the total 45 urine BKV

DNA positive HIV infected individuals, eight patients showed BK viral load >100 copies/ml. Of them, three treatment naïve patients were the individuals who showed highest BK viral load values than the other individuals. In specific, the author had also reported higher BKV positivity in patients receiving abacavir (*P* value 0.03) and efavirenz (*P* value 0.03) than other patients (14).

The percentage positivity of BK viremia in HAART experienced individuals to be much lower than treatment naïve HIV infected individuals as observed by Akhgari S *et al*, 2015. They reported about 4 out of 74 (5.4%) BK viremia in patients on ART when compared to 4 out of 25 (16%) in ART naïve patients (108). In our study, on follow up with individuals on ART, 6 out of 8 individuals had decrease or absence of BK virus following ART. When the BK viral load log was compared between treatment naïve and ART experienced there was no significant difference (*P* value=0.24) among the two groups. But the frequency of BK virus in the ART experienced group was lower emphasizing the role of ART in these individuals

To prove the opportunistic nature of the infectious agent, it is important to determine the frequency of these infections in normal healthy individuals. As BK virus is an opportunistic virus, its positivity is found to be much lower in healthy individuals than HIV infected individuals. This has been reported by various studies, a study from Colorado, USA, Markowitz *et al* (1992), showed that out of the 34 HIV negative healthy individuals, 17.6%

were positive for BK virus and the author has given a range of 8-18% viral shedding in urine in healthy controls (113).

Ledesma *et al* (2012) reported a significantly higher frequency of BK viruria in HIV infected individuals (51.7%) than healthy controls (21.7%) (*P* value 0.02) (14). Jin *et al* (1995) has not reported BK positivity in any of the healthy individuals tested. However the control group was smaller with only 18 individuals in this study (110). Gosert *et al* (2008), studied the frequency of BK virus in urine and plasma of 264 normal healthy blood donors. They found BKV positivity in 26 (9.8%) individuals only in their urine samples (53). There was no positivity with any of the healthy plasma samples. Behzad Behbahani et al (2004) reported the same finding as previously described studies as 10% of BKV positivity in urine of healthy controls against 25.5% in HIV infected individuals (111). Our study also depicts the same results as 10.7% in HIV negative healthy controls as compared to 25.6% in HIV infected individuals (*P* value 0.003) and no positivity with whole blood samples from HIV negative healthy controls as reported by Gosert *et al* (2008) (53)

Non-coding Control region (NCCR) is a unique region present in Polyomaviruses. This is coded by the late gene region of BK virus. This region has the origin of replication, transcription binding sites and promoter enhancing sequences which regulate the viral replication (114). This region is different from the conserved protein coding regions in that it is prone to rearrangements due to either duplication or deletion of a part or whole of the sequence blocks. Since the discovery of the virus, these rearrangements have been studied

by repeated passaging of the virus in various cell cultures, the process which itself makes it vulnerable for rearrangements (115). Such rearrangements are seen mostly in patients who have prolonged BK viremia with high viral loads in their blood. This is explained commonly in renal transplant patients. The exact mechanism of such rearrangements are unclear and various hypotheses have been documented. One such theory is based on the recombination between daughter and parent strands. BKV genome replicates in a bi directional manner starting from the origin of replication in the NCCR. During this process, the newly produced daughter strands and the unreplicated parent strand are in close proximity leading to recombination at heterologous sites (116). According to Gosert *et al* (2008), even the presence of mutations in this region when associated with unrestricted replication as seen in immunocompromised hosts, the proof reading capacity of the host cell machinery becomes futile leading on to major rearrangements. Moreover, the rearranged NCCR itself increases the expression of early gene region than archetype variant thus leading to higher rate of replication. The author also mentions about the association of NCCR rearrangements with the peak plasma viral load (*P* value <0.001) (53). All the above mentioned studies were done in the renal transplant settings. In a study done on HIV infected individuals, Markowitz *et al* (1992), out of the 26 BK positive urine samples, 6 samples had minor NCCR rearrangements showing deletion of a small NCCR segment of size 10-40bp and 4 samples showed an insertion with the product being longer than the archetype variant. These 4 samples with major rearrangements had severe degree of immunosuppression as evidenced by their CD4 counts which was <200cells/µl. Out of the 4, three (75%) of them had higher BK viral load then the other positive samples (204,

1142 and 8750 copies/ml) (113). Sundsfjord *et al* (1994) found rearrangements in 8 out of 22 BKV positive urine samples of which only 2 samples were confirmed by sequencing. Rearrangement reported in this study had a duplication of P block (50bp) which was the first type to be reported in clinical specimens during that period. The author also found no correlation between NCCR rearrangements and the degree of immunosuppression or viral load (107). Gosert *et al* (2008), a study done on renal transplant patients, revealed that 113(15%) out of 733 plasma samples from these patients showed rearranged NCCR with 11% showing insertions and 35% showing deletions. When they compared the frequency of rearrangements with the median BKV load, rearranged samples showed a significantly higher load (4.59×10^5 geq/ml) than samples showing archetype variant (1.94×10^4 geq/ml). Also when the authors examined BKV positive urine samples without concurrent viremia, they observed only archetype variants in these samples. They also found that the BKV NCCR sequences from urine of healthy blood donors also showed only an archetype variant (53) which matched with our study findings where the healthy control samples in our study did not show any rearrangements.

We had two urine samples from HIV infected individuals one from treatment naïve and other from an ART experienced individual showing NCCR rearrangements with duplication of a part of P (9bp) and Q blocks (23bp). There was no correlation either with degree of immunosuppression (CD4 counts) or with their BK viral load (29 and 72 copies/ml) These findings were in par with the findings of Sundsfjord *et al* (1994) (107). Two archived blood samples from HIV infected individuals retrieved from the study done

by Sachithanandham *et al* (2014) showed NCCR rearrangements. Both the individuals had higher HIV load (795041 and 266921copies/ml) as well as low CD4 counts (6 and 38 cells/µl) demonstrating the positive correlation between the presence of rearrangements and the degree of immunosuppression. Also both the individuals had a very high BK viral load (3000 and 17200 copies/ml). Thus NCCR rearrangements in blood correlates better with BK viral load than rearrangements seen in urine samples as mentioned by Gosert *et al* (2008) (53).

BKV Genotyping was done initially based on short sequence of VP1 using restriction fragment length polymorphism and DNA sequencing by Jin *et al* (1993) (117). Four genotypes were assigned by the authors which were not including all strains found in various samples subsequently. This led to the emergence of subgroups within these subtypes, as Ia and Ib first described by Stoner et al (2002) (118). The importance of genotyping still remains controversial as the exact pathogenic role of different genotypes are not well elucidated even though they have a role in epidemiological investigations and to know the origin of the virus and the evolving newer strains as described by Luo *et al* (2009) (49). However according to Nukuzuma *et al* (2006), BKV subtype I was found to replicate more efficiently in vitro in human kidney epithelial cells (119)

BKV genotype I is the most common genotype seen worldwide. Jin *et al* (1995) reported subtype I based on VP1 heterogeneity to be common in HIV infected individuals as they identified 22 out of 45 BKV DNA positive urine samples as genotype I (110). Genotyping based on NCCR has been done by Perets *et al* (2009). This was a study done on renal

transplant recipients where Ia, Ib1, Ib2, Ic, II, III and IV genotypes were described. The author has reported that genotyping based on NCCR sequences almost matches with the phylogenetic analysis done with VP1 region (12). Gosert *et al* (2008), genotyped a subset of rearranged NCCR samples to see the association between the NCCR rearrangements and the subtype determination. They found that irrespective of the rearrangements all the samples were identified as the same genotype as the archetype NCCR samples (53). In our study, we did genotyping based on NCCR sequences which revealed that all 44 samples genotyped were identified as genotype I. We also did genotyping based on VP1 region on 5 samples selected randomly and identified all samples as genotype I. This was to demonstrate the fact explained by Perets *et al* (2009) as there was almost no phylogenetic difference between genotyping based on NCCR sequences and VP1 region. The urine and blood samples irrespective of the NCCR rearrangements were also identified as genotype I in our study. This finding was the same as found in the literature published by Gosert *et al* (2008).

In conclusion, being this a first study on the prevalence of BK virus in HIV infected individuals in India, we found a higher frequency of BK viruria in HIV-1 infected individuals than HIV uninfected healthy controls. The BK viral load had a significant positive correlation with WHO clinical staging and a negative correlation with CD4 counts demonstrating the association of BKV viruria with the extent of immunosuppression as evidenced by other studies. The frequency and viral load of BKV observed among HIV

infected individuals is very low compared to what is reported in transplant patients following immunosuppression. We did not find any NCCR rearrangements to be associated with high BK viral load in urine or clinically severe disease as described in literature. However, individuals with NCCR rearrangements in blood sample had low CD4 counts and higher BK viral load. All samples which were genotyped based on NCCR and VP1 gene analysis were identified as genotype 1 which is the most common genotype worldwide as described. With the small subset of individuals on ART being followed up, it is clear that ART has a definite role in reducing the BKV load in these individuals.

7. Summary and conclusions

1. This study was done to quantitate BK virus in treatment naïve HIV-1 infected individuals and to correlate the viral load with degree of immunosuppression and WHO clinical staging.
2. The other objectives were to look for BKV NCCR rearrangements in these individuals and to determine the effectiveness of ART on BKV positivity by prospectively following a proportion of patients after a minimum of 3-6 months
3. Urine and whole blood samples from 187 treatment naïve HIV-1 infected individuals were tested for BK virus by a screening qualitative followed by quantitative real time PCR.
4. Forty six urine samples (25.6%) and 2 whole blood samples (1%) were positive for BKV DNA among the 187 HIV infected individuals.
5. The mean BK viral load in these 46 urine samples was 2.17 ± 1.5 $\log_{10}$ copies/ml.
6. The BK viral load of these samples showed significant negative correlation when compared with CD4 count (*P* value=0.02) by univariate analysis.
7. On the other hand, there was a positive correlation when BK viral load was compared with WHO clinical staging (*P* value < 0.001)
8. There was no significant correlation when BK viral load was compared with HIV load (*P* value 0.37)

9. Six out of eight individuals (75%) who were followed up after a minimum of 3 months of ART showed either a decrease in BK viral load (n=2) or negativity for BKV DNA (n=4)
10. The BK positivity in blood was very low (1%) that also with a very low viral load unlike reported in immunosuppression among transplant patients
11. We also studied the frequency of BKV in HIV negative healthy individuals, where 10 out of 93 (10.7%) individuals showed BKV DNA positivity in their urine with the mean viral load of 1.24±0.96 $\log_{10}$ copies/ml.
12. The percentage positivity of BKV among the healthy controls (10.7%) and that of HIV-1 infected individuals (25.6%) were significant (*P* value < 0.001).
13. There was a significant difference (*P* value=0.02) between the BKV load of HIV-1 infected individuals and healthy controls.
14. Out of the 42 urine samples sequenced for BKV NCCR, 2 urine samples, both from HIV-1 infected individuals, one from treatment naïve and other from a follow up individual after ART showed rearrangements. A part of P (9bp) and Q (23bp) blocks of NCCR were duplicated in these samples. All the other 40 samples showed archetype NCCR sequence.
15. Two archived blood samples positive for BKV DNA in HIV-1 infected individuals from our previous study also showed two different patterns of NCCR rearrangements. Almost the whole of P (60bp) and Q blocks (35bp) was duplicated in one sample and the other sample showed duplication of a part of P (51bp) and Q

(28bp) blocks. This was done to look for rearrangements in blood, as the present study's whole blood samples could not be amplified in terms of low viral load.

16. NCCR rearrangements were not found to be associated with high BK viral load in urine or clinically severe disease as described in literature.
17. However, individuals with NCCR rearrangements in blood sample had low CD4 counts and higher BK viral load.
18. All samples (n=44) which were genotyped based on NCCR sequences were identified as genotype I and this was confirmed by genotyping 5 randomly selected samples with VP1 based genotyping.

Limitation of the study: More number of HIV-1 infected individuals after ART need to be followed up to determine the true impact of HAART on declining the BK viral load or on complete clearance of the virus.

Conclusion:

In conclusion, being this a first study on the prevalence of BK virus in HIV infected individuals in India, we found that the frequency of BK viruria as well as the mean viral load are higher in HIV-1 infected individuals than HIV negative healthy individuals. Unlike in transplant patients with immunosuppression as reported, the viremia is negligible among HIV infected individuals. The BKV DNA load has a significant negative correlation with their CD4 counts and significant positive correlation with

WHO clinical staging. This proves that there is a significant correlation between the extent of immunosuppression and BKV viruria as evidenced by other studies. We did not see any NCCR rearrangements to be associated with high BK viral load in urine or clinically BK virus specific severe disease as described in literature. However, individuals with NCCR rearrangements in blood sample had low CD4 counts and higher BK viral load. All samples which were genotyped based on NCCR and VP1 gene analysis were identified as genotype 1 which is the most common genotype worldwide as described. With the small subset of individuals on ART being followed up, it is clear that ART has a definite role in reducing the BKV load in these individuals.

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9. Annexure

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

September 24, 2014

Dr. V. J. Subha
PG Registrar
Department of Clinical Microbiology
Christian Medical College, Vellore 632 004

Sub: **Fluid Research Grant Project:**
Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression.
Dr. V. J. Subha, Clinical Microbiology, Dr. Rajesh Kannangai, Dr. Asha Mary Abraham, Clinical Virology, Dr. George M. Varghese, Dr. Anand Zachariah, Medicine, Mr. Jaiprasath, Mr. John Paul Demosthenes, Clinical Virology, CMC, Vellore.

Ref: IRB Min No: 8982 [DIAGNOSE] dated 04.08.2014

Dear Dr. V. J. Subha,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Rajesh Kannangai, Clinical Virology, CMC, Vellore

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

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September 24, 2014

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Ref: IRB Min No: 8982 [DIAGNOSE] dated 04.08.2014

Dear Dr. V. J. Subha,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression." on August 4th 2014.

The Committees reviewed the following documents:

1. IRB Application format
2. Curriculum Vitae' of Drs. V. J. Subha, Rajesh Kannangai, Asha Mary Abraham, George M. Vargheese, Anand Zachariah, Jaiprasath, John Paul Demosthenes
3. Informed Consent form (English, Tamil, Telugu, Hindi & Bengali)
4. Information Sheet (English, Tamil, Telugu, Hindi & Bengali)
5. No of documents 1-4

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on August 4th 2014 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
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Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Name	Qualification	Designation	Other Affiliations
Dr. Benjamin Perakath	MBBS, MS, FRCS	Professor, Colorectal Surgery, CMC, Vellore	Internal, Clinician
Dr. Rajesh Kannangai	MD, Ph D.	Professor & In-charge Retrovirus Laboratory (NRL under NACO), Department of Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Anup Ramachandran	Ph. D	The Wellcome Trust Research Laboratory Gastrointestinal Sciences, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Simon Pavamani	MBBS, MD,	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. Vivek Mathew	MD (Gen. Med.) D.M (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC, Vellore	Internal, Clinician
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Dr. Bobby John	MBBS, MD, DM, Ph D, MAMS	Professor, Cardiology, CMC, Vellore	Internal, Clinician
Dr. Chandrasingh	MS, MCH, DMB	Professor, Urology, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Dept of Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Inian Samarasam	MS, FRCS, FRACS	Professor, Surgery, CMC, Vellore	Internal, Clinician

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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. B. J. Prashantham	MA(Counseling Psychology), MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Mrs. Pattabiraman	B. Sc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. Jayaprakash Muliyil	B. Sc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, CMC, Vellore	External, Scientist &Epidemiologist
Dr. Denise H. Fleming	B. Sc (Hons), PhD	Honorary Professor, Clinical Pharmacology, CMC, Vellore	Internal, Scientist & Pharmacologist
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mrs. Sheela Durai	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mr. C. Sampath	BSc, BL	Legal Expert, Vellore	External, Legal Expert
Dr. Anuradha Rose	MBBS, MD	Assistant Professor, Community Health, CMC, Vellore	Internal, Clinician
Dr. Nihal Thomas,	MD, MNAMS, DNB(Endo), FRACP(Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. Additional Vice Principal (Research), Deputy Chairperson, IRB, Member Secretary (Ethics Committee), IRB, CMC, Vellore	Internal, Clinician

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
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MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Fluid Grant Allocation:

A sum of 75,000/- INR (Rupees Seventy Five Thousand only) will be granted for 18 months.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Rajesh Kannangai, Clinical Virology, CMC, Vellore

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OFFICE OF RESEARCH
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Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

August 12, 2014

Dr. V. J. Subha
PG Registrar
Department of Clinical Microbiology
Christian Medical College
Vellore 632 004

Sub: **Fluid Research Grant Project:**
Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression.
Dr. V. J. Subha, Clinical Microbiology, Dr. Rajesh Kannangai, Clinical Virology, Dr. Asha Mary Abraham, Clinical Virology, Dr. George M. Vargheese, Medicine Unit, Dr. Anand Zachariah, Medicine, Mr. Jaiprasath, Clinical Virology, Mr. John Paul Demosthenes, Clinical Virology, CMC, Vellore.

Ref: IRB Min No: 8982 dated 04.08.2014

Dear Dr. V. J. Subha,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression." on August 4th 2014. I am quoting below the minutes of the meeting.

The Committee raised the following queries:

a) What is the standard care for haemorrhagic cystitis?
b) The information sheet that is provided is too general in nature, and needs to be modified.
c) There is something about a clinical trial in the informed consent form-this can be removed.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
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OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. V. J. Subha and Dr. Rajesh Kannangai, were present during the presentation of the proposal and satisfactorily responded to the queries raised by the Members. After discussion, it was resolved to **ACCEPT the proposal AFTER receiving the suggested modifications and answers to the queries.**

Note:
1. Kindly **HIGHLIGHT** the modifications in the revised proposal.
2. Keep a **covering letter and point out the answer to the queries.**
3. Reply to the queries should be submitted within **3 months** duration from the time of the thesis/ protocol presentation, if not the thesis/protocol have to be resubmitted to the IRB.
4. The **checklist** has to be sent along with the responses.

Email the details to research@cmcvellore.ac.in and send a hard copy through internal dispatch to Dr. Nihal Thomas, Addl. Vice Principal (Research), Principal's Office, CMC.

Yours sincerely,

CHRISTIAN MEDICAL COLLEGE VELLORE INDIA

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

CC: Dr. Rajesh Kannangai, Professor, Department of Clinical Virology, CMC

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OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD
CHRISTIANMEDICALCOLLEGE,
BAGAYAM, VELLORE 632002, TAMIL NADU, INDIA

Ref: FG/8982/08/2014

October 08, 2014

Mr. Robby Pria Sundersingh
The Treasurer
Christian Medical College,
Vellore.

Dear Mr. Robby Pria Sundersingh,

Sub: **Fluid Research Grant Project:**
Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression.
Dr. V. J. Subha, Clinical Microbiology, Dr. Rajesh Kannangai, Dr. Asha Mary Abraham, Clinical Virology, Dr. George M. Vargheese, Dr. Anand Zachariah, Medicine, Mr. Jaiprasath, Mr. John Paul Demosthenes, Clinical Virology, CMC, Vellore.

Ref: IRB Min. No. 8982 dated 04.08.2014

The Institutional Review Board at its meeting held on August 04th 2014 vide IRB Min. No. **8982** accepted the project for a sum *75,000/- INR (Rupees Seventy Five Thousand only) will be granted for 18 months. If overspent the excess should be debited form the respective departmental or Special funds.*Kindly arrange to transfer the sanctioned amount to a separate account to be operated Dr. V. J. Subha and Dr. Rajesh Kannangai.

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD,MNAMS,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

CC: Dr. V. J. Subha, Clinical Microbiology, CMC. Vellore
Dr. Rajesh Kannangai, Clinical Virology, CMC, Vellore
File

PATIENT INFORMATION SHEET

Study title: Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression.

Please read this carefully. It tells you important information about the study. A member of the research team will explain you about your participation in this study. If you have any questions about the research or about this form, please ask us. If you decide to take part in this study, you must sign or provide your thumb impression in this form to show your willingness to take part in this study.

Why is this study being done?

BK virus is an important cause of various diseases in persons infected with HIV/AIDS. This virus infects individuals when they are very young through respiratory tract and at that time the virus will not cause any disease. However, this virus will remain in the kidneys without multiplication. When the immunity of a person goes down, this virus gets reactivated and cause disease mainly affecting the kidneys. HIV is a viral infection which causes severe immunosuppression (lowers immunity). So this condition predisposes the individual to various infections and one among that is BK virus associated kidney disease. So in this study we are going to look at the amount (quantitation) of BK virus in blood and urine of HIV infected individuals and its association with the clinical manifestations. We will also look at the presence of this virus in healthy adults (controls).

What will happen in this study?

Individuals above 18 years, either sex, who are serologically positive for HIV but not on treatment and 85 healthy individuals who are HIV negative, will be recruited in this study.

If you agree to participate and you meet all the criteria required for the study either as patient/control, blood and urine samples will be collected from you to test for the presence of BK virus.

Basic information, medical history and details of treatment will be collected from you.

Will I be paid to take part in this study?

You will not be paid for providing your sample for this research.

What are the risks and possible discomforts from being in this study?

Blood collection is done routinely for CD4+ T cell count estimation and at that time an additional 6ml of blood will be collected. No additional risk is associated with this procedure. Urine samples will be collected from you in the Department of Clinical Virology, with your consent to participate in the study.

What are the possible benefits from being in this study?

By being a part of this study, you will not benefit directly with regard to the immediate management of the disease, but as a result of your participation, we will be able to confirm the presence or absence of the virus under study and the response of this viral infection to HIV treatment. If you are found positive for BK virus in blood you can be referred to the Department of Nephrology for standard care.

If I take part in this research study, how will you protect my privacy?

Information collected from you for this research study will be stored in the investigator's research files. Your name and other information that might identify you will be recorded with a unique code number, protecting your identity and information from others. The research consent form that you sign may be inspected by the regulatory agencies or the Institutional Review Board in the course of carrying out their duties. If the signed research consent form is inspected or copied, the hospital will use reasonable efforts to protect your privacy. The results obtained from this study will be published in the scientific journals, but no information about your identity will be disclosed.

If I have questions or concerns about this research study, whom can I call?

You can contact the following person for your questions about the study

Dr.V.J.Subha
PG Registrar
Department of Microbiology, Christian Medical College, Vellore - 632004

Phone: 0416 2282588, 9486082377

INFORMED CONSENT FORM

Study Title: Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression

Study Number: _____

Subject's Name: _____

Date of Birth / Age: _____

(Subject)

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions.

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

(iii) I understand that the Sponsor of the clinical trial, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published.

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).

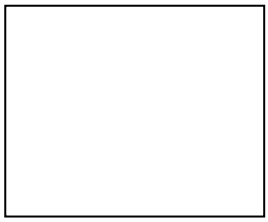
(v) I agree to take part in the above study.

Signature (or Thumb impression) of the Subject/Legally Acceptable

Date: _____/_____/_____

Signatory's Name: _____ Signature:

Or



Representative: _____

Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____

Date: _____/_____/_____

Study Investigator's Name: _____

Signature of the Witness: _____

Date: _____/_____/_____

Name and Address of the Witness: _____

PATIENT PROFORMA

Study Title: Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression

Study number: **Date:**

Name:

Hospital number:

Date of birth / Age: **Sex:**

Address:

State:

Mother tongue:

Marital Status:

Clinical diagnosis: WHO I/II/III/IV - will be done by the physician (Co- investigator)

Date of HIV diagnosis:

History of renal disease

Symptoms: Decreased urine output

Macroscopic Hematuria

Pedal oedema

Dysuria

Suprapubic / flank pain

Myalgia

Already diagnosed renal disease:

If yes**, Cause -**

Mode of diagnosis -

History of organ transplantation:

History of chronic diarrhoea

Weight loss >1m

Fever

Cough

History/Diagnosed OI/other infections (This information will be obtained retrospectively from the clinical workstation/patient work chart/discussion with the clinician about the patient for WHO classification):

a)Tuberculosis, b)Candidiasis, c)Herpes zoster, d)*Pneumocystis carinii* pneumonia, e)Bacterial pneumonia, f)Salmonellosis, h)Toxoplasmosis, i)Cryptosporidiosis, j)CMV, k)Cutaneous Fungal infection, l)Pelvic Inflammatory Disease, m)Cryptococcal meningitis, n)Histoplasmosis, o)Hepatitis

Follow up

Date:

CD4+/CD8+ T cell count:

History/Diagnosed other infections:

Sample collected by

Name:

Signature:

PROFORMA FOR HIV TESTING

Study title: Virological characterization of BK virus among HIV-1 infected individuals and its association with immunosuppression.

Consent Form for HIV Testing

This is to state that I have been counseled about the HIV testing and details about HIV have been explained to me in a manner that I can understand, I, hereby give my consent for the test to be conducted in me / my _____ in order to ascertain my / _____ HIV Sero status

எச். ஐ.வி. பரிசோதனை ஒப்புதல் படிவம்

HIV பரிசோதனை குறித்து ஆலோசனைகளும் HIV பற்றின விவரங்களும் நான் புரிந்து கொள்ளும் வகையில் விளக்கி கூறப்பட்டது என் / எனது _____ HIV நோயை அறிவதற்காகத்தான் பரிசோதனை செய்யப்பட்டது என்று அறிந்து ஒப்புதல் அளிக்கிறேன்.

स्वीकृथी पत्र एच.ई.वि जांच के लिए

यह प्रमाणिक किया जाता है की मुझे एच.ई.वि जांच के बारे में मेरे समजने योग्य तरीके से, विस्तार पूर्वक संजय किया गया है. मै / मेरे _____ पर/ इस जांच के लिए स्वीकारनी देता / देती हूँ

এইচ আই ভি পরীক্ষার জন্য সম্মতি ফর্ম

আমি সত্য বলিয়া ঘোষণা করিতেছি যে আমাকে এইচ আই ভি পরীক্ষা সম্বন্ধে পরামর্শ দেওয়া হইয়াছে এবং এইচ আই ভি সম্বন্ধে বিশদ বর্ণনা আমাকে আমার বোঝার মতো করে জানানো হইয়াছে। আমি এখন, আমার উপরে/ আমার.....উপরে আমার/..... এইচ আই ভি সেরোস্ট্যাটাস সম্বন্ধে বিশদ জানার জন্য যে পরীক্ষা হইবে তাহার সম্মতি দিতেছি।

Signature and Date கையொப்பம் / தேதி ಸಹಿ/ದಿನಾಂಕ हस्ताक्षर/तारिक স্বাক্ষর এবং তারিখ

Details of HIV-1 infected individuals

HBK No.	Age	Sex	Qualitative BK result	CD4 count	WHO stage	Geographic region	BK VL	HIV-1 VL
1	33	F	negative	540	1	tamilnadu		0
2	36	M	negative	777	1	west bengal		71
3	36	F	negative	465	2	west bengal		2987
4	28	M	negative	204	1	andaman & nicobar		4942
5	46	M	positive	444	3	tamilnadu	43,240	80574
6	29	F	negative	1006	1	tamilnadu		649
7	41	F	negative	722	1	westbengal		95
8	54	M	positive	537	3	andhrapradhesh	8	71
9	35	F	negative	442	1	tamilnadu		4986
10	39	F	negative	711	1	kerala		488
11	46	M	negative	620	1	tamilnadu		1363
12	36	F	negative	546	3	tamilnadu		3491
13	32	M	negative	694	1	tamilnadu		2209
14	32	F	positive	584	2	tamilnadu	359886	1422
15	32	M	positive	362	1	andhrapradhesh	1	828
16	26	M	negative	297	1	tamilnadu		330
17	35	M	negative	93	3	tamilnadu		19598
18	60	F	negative	100	3	tamilnadu		290974
19	34	F	negative	769	3	andhra pradhesh		71
20	37	M	negative	142	4	andhra pradhesh		5913
21	34	F	positive	459	1	tamilnadu	1421	1596
22	49	M	positive	261	4	tamilnadu	1	12972
23	37	F	negative	677	1	tamilnadu		71
24	30	F	negative	630	1	tamilnadu		71
25	55	M	negative	545	1	tamilnadu		53399
26	32	M	positive	133	1	tamilnadu	9	740312
27	58	M	negative	281	2	tamilnadu		108
28	23	M	negative	755	1	andhra pradhesh		96698
29	46	M	positive	515	2	tamilnadu	3	100816
30	25	M	negative	323	1	tamilnadu		2441
31	31	F	negative	397	1	tamilnadu		3341
32	38	F	positive	1043	1	andhrapradhesh	4	5845
33	37	F	negative	351	2	west bengal		6226
34	38	M	negative	116	1	jharkhand		340912
35	50	M	positive	391	3	andhrapradhesh	110	489718
36	61	M	negative	794	1	tamilnadu		1364
37	48	M	negative	517	3	tamilnadu		33419
38	53	M	negative	641	1	orissa		0
39	23	M	negative	593	1	tamilnadu		6004
40	26	M	positive	24	4	andhrapradhesh	326	385083
41	51	M	negative	457	1	tamilnadu		36727
42	30	F	negative	584	1	tamilnadu		1010
43	40	M	negative	314	1	tamilnadu		376
44	39	F	negative	330	4	tamilnadu		862329
45	35	F	negative	611	1	tamilnadu		301
46	40	F	negative	142	3	tamilnadu		45393
47	43	F	negative	224		andhra pradhesh		9754

HBK No.	Age	Sex	Qualitative BK result	CD4 count	WHO stage	Geographic region	BK VL	HIV-1 VL
48	32	F	negative	912	1	andhra pradhesh		1616
49	57	M	negative	457	1	tamilnadu		3600
50	47	M	negative	591	1	andhra pradhesh		5076
51	41	M	negative	398	1	andhra pradhesh		37848
52	42	M	negative	529	1	andhra pradhesh		369
53	45	M	negative	340	4	andhra pradhesh		1450
54	32	F	negative	509	1	tamilnadu		273
55	44	F	negative	874	1	tamilnadu		69
56	41	F	negative	76	4	west bengal		33230
57	42	M	negative	354	1	tamilnadu		28800
58	34	M	positive	48	4	jharkhand	1717	16133
59	41	F	negative	582	1	tamilnadu		755
60	46	F	negative	315	1	tamilnadu		73229
61	22	M	positive	31	4	andhrapradhesh	11	358
62	61	M	negative	779	1	andhra pradhesh		43310
63	37	M	negative	256	1	tamilnadu		19643
64	40	M	negative	434	1	tamilnadu		26718
65	36	F	negative	675	1	tamilnadu		4021
66	50	M	negative	331	1	west bengal		448506
67	41	M	negative	455	4	tamilnadu		448506
68	32	F	negative	404	1	tamilnadu		3995
69	39	F	negative	1300	1	tamilnadu		71
70	37	F	negative	480	1	tamilnadu		445
71	29	F	negative	790	1	tamilnadu		2107
72	41	F	positive	222	4	andhrapradhesh	88	5115
73	31	M	positive	57	3	andhrapradhesh	886	18256
74	68	M	positive	527	1	tamilnadu	1	1494
75	31	M	negative	459	3	tamilnadu		13488
76	58	M	positive	41	4	tamilnadu	6018	460
77	42	M	positive	529	4	andhrapradhesh	27837	0
78	56	F	positive	533	1	andhrapradhesh	neg	616
79	29	F	negative	140	4	andhra pradhesh		
80	39	F	negative	376	1	tamilnadu		
81	38	F	negative	329	2	tamilnadu		
82	58	F	negative	392	2	tamilnadu		
83	49	M	negative	301	3	andhra pradhesh		
84	47	M	negative	152	4	tamilnadu		
85	45	M	negative	66	4	madhya pradhesh		
86	30	M	negative	238	2	tamilnadu		
87	39	F	negative	1176	1	andhra pradhesh		
88	34	F	negative	412	1	tamilnadu		
89	46	F	negative	375	2	tamilnadu		
90	45	M	positive	295	1	tamilnadu	1	854028
91	34	M	positive	46	4	assam	582	1051785
92	33	F	negative	713	1	tamilnadu		
93	34	F	negative	565	1	tamilnadu		
94	32	F	negative	756	1	tamilnadu		

Details of HIV-1 infected individuals

HBK No.	Age	Sex	Qualitative BK result	CD4 count	WHO stage	Geographic region	BK VL	HIV-1 VL
95	32	M	negative	436	3	tamilnadu		
96	35	F	negative	117	2	jharkhand		
97	44	M	positive	18	4	andhrapradhesh	174	1465719
98	42	M	negative	190	3	west bengal		
99	40	F	negative	486	1	tamilnadu		
100	29	M	negative	327	1	tamilnadu		
101	28	M	negative	443	1	west bengal		
102	42	F	negative	785	1	andhra pradhesh		
103	37	M	positive	343	1	andhrapradhesh	477	43436
104	52	F	negative	667	1	andhra pradhesh		
105	40	M	negative	176	3	andhra pradhesh		
106	34	F	negative	577	3	andhra pradhesh		
107	59	M	negative	359	1	andhra pradhesh		
108	36	F	negative	377	1	tamilnadu		
109	30	M	negative	167	2	tamilnadu		
110	38	F	positive	455	1	tamilnadu	73	21461
111	35	F	positive	31	4	tamilnadu	31	3533
112	58	M	negative	29	3	bihar		
113	35	F	negative	590	1	tamilnadu		
114	45	M	negative	479	1	andhra pradhesh		
115	41	M	negative	49	4	tamilnadu		
116	36	M	positive	177	1	tamilnadu	1	532936
117	42	F	negative	610	1	tamilnadu		
118	56	M	negative	43	2	west bengal		
119	35	F	positive	697	1	tamilnadu	29	87
120	39	M	positive	72	3	tamilnadu	26882	267901
121	36	F	positive	663	3	andhrapradhesh	95645	11937
122	35	M	negative	271	3	tamilnadu		
123	34	M	negative	437	1	tamilnadu		
124	37	F	negative	483	1	tamilnadu		
125	44	M	positive	540	1	tamilnadu	1	1942
126	59	M	negative	636	2	tamilnadu		
127	32	F	negative	631	1	tamilnadu		
128	37	M	positive	312	1	andhrapradhesh	134	117637
129	30	M	positive	455	1	tamilnadu	0	133801
130	52	M	negative	31	4	tamilnadu		
131	53	M	negative	501	3	andhra pradhesh		
132	44	M	negative	25	4	west bengal		
133	55	M	negative	521	3	tamilnadu		
134	31	F	negative	502	1	tamilnadu		
135	46	F	negative	441	1	tamilnadu		
136	45	F	negative	791	1	tamilnadu		
137	35	M	negative	665	3	tamilnadu		
138	37	M	positive	332	2	andhrapradhesh	268	20833
139	54	M	negative	55	2	tamilnadu		
140	26	M	negative	28	3	west bengal		
141	30	F	negative	28	3	tamilnadu		

HBK No.	Age	Sex	Qualitative BK result	CD4 count	WHO stage	Geographic region	BK VL	HIV-1 VL
142	46	M	positive	75	4	bihar	4	164535
143	41	M	negative	734	1	andhra pradhesh		
144	50	F	negative	740	1	tamilnadu		
145	63	M	negative	221	1	andhra pradhesh		
146	26	M	negative	432	1	tamilnadu		
147	33	M	positive	310	3	tamilnadu	19	71
148	44	M	positive	471	1	andhrapradhesh	4474	1709
149	36	F	positive	274	1	andhrapradhesh	72	25676
150	28	F	negative	332	1	jharkhand		
151	38	F	negative	562	1	kerala		
152	38	F	positive	665	1	tamilnadu	86	71
153	56	F	negative	410	1	tamilnadu		
154	40	F	negative	294	3	andhra pradhesh		
155	41	F	positive	197	1	andhrapradhesh	185	486003
156	35	F	negative	92	1	tamilnadu		
157	32	M	negative	871	1	tripura		
158	42	F	negative	414	1	tamilnadu		
159	32	M	negative	594	1	andhra pradhesh		
160	42	F	negative	515	1	andhra pradhesh		
161	27	F	negative	445	1	tamilnadu		
162	48	F	negative	481	1	andhra pradhesh		
163	37	M	positive	459	1	tamilnadu	87368	2306
164	37	M	negative	637	1	tamilnadu		
165	38	M	negative	7	4	tamilnadu		
166	49	M	negative	tube failure	3	tamilnadu		
167	38	M	negative	769	1	andhra pradhesh		
168	39	F	negative	337	1	tamilnadu		
169	41	M	positive	48	4	tamilnadu	1171	5888659
170	30	M	negative	140	3	west bengal		
171	64	M	negative	462	1	chattisgarh		
172	42	M	positive	45	4	tamilnadu	1615	not done
173	35	F	negative	116	1	tamilnadu		
174	25	F	positive	613	1	tamilnadu	74	12948
175	44	F	positive	660	1	tamilnadu	11888	62905
176	38	F	negative	47	2	andhra pradhesh		
177	32	M	negative	398	1	tamilnadu		
178	28	M	negative	324	1	west bengal		
179	28	F	positive	719	1	tamilnadu	2	81293
180	34	F	negative	728	1	tamilnadu		
181	61	M	positive	451	1	tamilnadu	55	9001
182	46	F	negative	495	1	tamilnadu		
183	46	M	negative	64	4	tamilnadu		
184	44	M	positive	28	4	tamilnadu	184	39437
185	47	F	negative	307	1	tamilnadu		
186	49	M	negative	701	1	tamilnadu		
187	40	F	positive	206	1	tamilnadu	93	21638

Details of HIV uninfected healthy controls

HBKC No	Age	Sex	BK qualitative	BK viral load
1	28	F	negative	
2	27	M	negative	
3	27	M	negative	
4	27	M	negative	
5	27	F	negative	
6	23	M	negative	
7	36	F	negative	
8	43	F	negative	
9	50	M	negative	
10	38	M	negative	
11	26	F	negative	
12	30	F	negative	
13	23	M	negative	
14	28	M	negative	
15	29	M	negative	
16	42	M	negative	
17	32	F	negative	
18	32	F	negative	
19	30	F	negative	
20	33	M	negative	
21	31	F	negative	
22	56	F	negative	
23	33	F	negative	
24	33	M	negative	
25	26	F	negative	
26	34	M	positive	2
27	44	M	negative	
28	41	M	negative	
29	44	M	negative	
30	44	F	negative	
31	41	M	negative	
32	39	F	negative	
33	32	M	negative	
34	38	M	negative	
35	42	F	negative	
36	29	F	negative	
37	55	F	negative	
38	53	M	negative	
39	46	F	negative	
40	43	F	negative	
41	42	M	negative	
42	46	F	negative	
43	42	F	negative	
44	45	M	negative	
45	45	M	negative	
46	47	F	positive	7
47	35	F	negative	

HBKC No	Age	Sex	BK qualitative	BK viral load
48	41	F	negative	
49	41	F	negative	
50	39	F	positive	2
51	42	M	positive	34
52	40	M	negative	
53	53	F	negative	
54	39	F	negative	
55	49	M	negative	
56	43	M	positive	9
57	40	M	negative	
58	46	M	negative	
59	49	M	negative	
60	36	F	negative	
61	38	F	negative	
62	37	F	negative	
63	29	M	positive	240
64	36	M	negative	
65	40	M	negative	
66	35	F	negative	
67	35	F	negative	
68	37	M	negative	
69	37	F	negative	
70	32	M	negative	
71	40	M	negative	
72	31	M	negative	
73	37	M	negative	
74	47	M	negative	
75	34	M	negative	
76	41	M	negative	
77	35	F	positive	1
78	35	M	negative	
79	34	F	negative	
80	37	M	negative	
81	47	M	negative	
82	26	M	negative	
83	38	F	negative	
84	34	M	negative	
85	39	M	negative	
86	47	F	negative	
87	62	M	negative	
88	44	F	negative	
89	49	M	negative	
90	35	F	negative	
91	43	F	positive	572
92	39	F	positive	12
93	28	F	positive	192